Hematopoietic Stem Cell Maintenance in Steady-State and Inflammation

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ABBREVIATIONS

5-FU	5 Fluorouracil
AGM	aorta-gonadmesonepros region
AML	Acute myeloid leukemia
Ang-1	Angiopoietin 1
BM	Bone marrow
BrdU	Bromodeoxyuridine
CAR cells	CXCL12 abundant reticular cells
CB	Cord blood
CD	Cluster of differentiation
cDNA	complementary DNA
CFSE	5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester
CFU-E	Colony- forming unit - erythrocyte
CFU-F	Colony- forming unit – fibroblast
CFU-G	Colony- forming unit – granulocyte
CFU-GM	Colony- forming unit – granulucyte macrophage
CFU-GEMM	Colony- forming unit – granulucyte erythrocyte macrophage megakaryocyte
CFU-M	Colony- forming unit – macrophage
CFU-Mk	Colony- forming unit – megakaryocyte
c-kit	c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (Stem cell growth factor receptor)
CLP	Common lymphoid progenitor cells
CMP	Common myeloid progenitor cells
CXCL12	C-X-C motif chemokine ligand 12 (SDF-1)
DNA	Desoxyribonucleic acid
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FL	Fetal Liver
Flt3L	FMS-like- tyrosine kinase ligand
G0	Gap 0 cell cycle phase
G1	Gap 1 cell cycle phase
G-CSF	Granulocyte colony stimulating factor

GMP	Granulocyte-macrophage progenitor
GM-CSF	Granulocyte macrophage colony stimulating factor
H2AX	H2A histone family member X
H&E	haematoxylin and eosin
HHLS	Human hemato-lymphoid system
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IFNα/γ	Interferon alpha/gamma
IL-6	Interleukin 6
KI	Knock-in
LepR	Leptin receptor
LIC	leukemia-initiating cell
Lin-	Lineage negative
LKS	Lin-c-kit+Sca-1+
LMPP	Lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharide
LT-HSC	Long-term hematopoietic stem cell
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythrocyte progenitor
MHC	Major histocompatibility complex
MLP	Multipotent lymphoid progenitor
mPCL	Medical-grade polycaprolactone
MPP	Multipotent progenitor
MSC	Mesenchymal stromal cell (or Mesenchymal stem cell)
MyD88	Myeloid differentiation primary response gene 88
MyelP	Myeloid progenitor
NAC	N-acetyl-L-cysteine
NaS	Sodium salicylate
NBD	NEMO-binding domain peptide
NK cells	Natural killer cells
NSG	NOD scid gamma

PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Sca-1	Ly6a lymphocyte antigen 6 complex
SCF	Stem cell factor
SDF-1α	Stromal cell-derived factor 1 alpha
SEC	Sinusoidal endothelial cells
S/G2/M	Synthesis/ Gap2/ Mitosis
ST-HSC	Short-term hematopoietic stem cell
Tg	Transgene
TGF-β	Transforming growth factor- beta
Tie2	Tyrosine kinase with immunoglobulin-like and EGF-like domain 2 (or angiopoietin receptor)
TIFAB	TRAF-interacting protein with forkhead-associated domain B
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TPO	Thrombopoietin
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tx	Transplantation
UC	Umbilical cord
WAT	White adipose tissue
Wnt	Wingless oncogene analog
WT	Wild type

SUMMARY

Blood cells in the hematopoietic system are derived from lifelong self-renewing hematopoietic stem cells (HSC) in the bone marrow (BM). The two major characteristics of HSCs are their ability to self-renew and to generate all lineage cells of blood and the immune system throughout the lifespan of an organism. As the majority of the mature hematopoietic cells have a relatively short half-life, mature blood cells need to be constantly generated by the upstream precursors. Nevertheless, in case of hematopoietic stress, such as bleeding or infection/inflammation, the hematopoietic system is capable of rapid adaption by increasing cellular output in order to meet the higher demand of the respective blood cell type production. In case of infection or inflammation, non-hematopoietic and hematopoietic cells as immune cells and hematopoietic stem and progenitors cells, recognize the invading pathogen by pattern recognition receptors (PRR). Toll-like receptors (TLR), a group of PRRs, recognize microbial products derived from exogenous pathogens and induce a cell-dependent proliferation, differentiation, migration, as well as protein production and secretion in order to generate an efficient immune response against invaliding pathogens. Recent findings have indicated that not only the peripheral mature immune cells but also HSCs in BM can respond to infection by sensing pathogen-associated molecular patterns (PAMPs) through PRRs or pro-inflammatory cytokines through respective cytokine receptors.

We previously showed that in vivo repetitive challenge with lipopolysaccharide (LPS), recapitulating gram-negative bacterial infection through Toll-like receptor 4 (TLR4) stimulation, drives dormant HSCs into proliferation[1]. Therefore, in one part of my thesis, I addressed the consequences for HSC function upon LPS challenge and the underlying molecular mechanisms. This study shows that direct activation of TLR4 limits the competitive repopulating ability of HSCs via TRIF, but not MYD88-mediated pathways, and increases divisional history and DNA damage and repair responses in HSCs. *In vivo* pharmacological inhibition of reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (MAPK) inhibits LPS-induced HSC proliferation and dysfunction, identifying ROS-p38 as the responsible TLR4 downstream signals. These findings suggest that systemic infection might cause accumulation of genetic events in HSCs that eventually even might lead to malignant transformation and eventually development of leukemia.

Hematopoietic homeostasis is maintained in a specialized microenvironment in the BM, the so-called BM HSC niche. The cellular property of hematopoietic stem cells (HSCs), i.e., self-renewal and differentiation, is tightly controlled to sustain lifelong blood production via extrinsic factors supplied in part by BM niche cells. While the cellular and molecular components of the mouse BM niche have been extensively studied in experimental animals, little is known about human BM niche components.

In the second part of my thesis, I took a developmental tissue engineering approach that allows to *ex vivo* generate a human cartilage template with human adult BM-derived mesenchymal stromal cells (MSCs), and to *in vivo* develop human bone organs, thereafter called “ossicles” through endochondral ossification, to study human BM niche homeostasis. *Ex vivo* developed ossicles were implanted into immune-deficient human cytokine knock-in mice which were transplanted with cord blood (CB)-derived human CD34+ cells in order to reconstitute human hematopoiesis, following sublethal irradiation. This study indicates that human MSC-derived ossicles develop a vascular network and a mature trabecular bone-like structure with the development of functional human hematopoiesis with phenotypic HSCs. This suggests that human ossicles can serve as niche to support human hematopoiesis. Furthermore, a successful reengineering of the humanized BM niche by overexpressing SDF-1α, a chemokine protein responsible for the maintenance of HSCs, shows an increase in functional human HSCs and progenitor engraftment and maintenance.

These findings indicate that bone organs, *in vivo* developed by adult BM MSCs, can support engraftment and maintenance of allogeneic human HSCs and hematopoiesis, proving a functional human BM niche. The engineering of a heterotrophic human BM niche that is transplantable and genetically re-engineerable will serve as a platform that allows to study physiology and pathophysiology of healthy and diseased human hematopoiesis in an optimized environment *in vivo*.

ZUSAMMENFASSUNG

Der Ursprung aller Blutzellen im hämatopoetischen System stammt aus sich lebenslang selbst-erneuernden hämatopoetischen Stammzellen (HSC) im Knochenmark (BM). Die zwei Hauptmerkmale von HSC sind ihre Fähigkeit, sich selbst zu erneuern und alle Zelllinien des Blutes und des Immunsystems während der Lebensdauer eines Organismus zu erzeugen. Da die meisten reifen hämatopoetischen Zellen eine relativ kurze Halbwertszeit aufweisen, müssen reife Blutzellen ständig durch die vorgeschalteten Vorläufer erzeugt werden. Bei hämatopoetischem Stress, wie Blutungen oder Infektionen / Entzündungen, das hämatopoetische System durch Erhöhung der Zellproduktion schnell angepasst werden, um den höheren Bedarf der jeweiligen Blutzellen zu erfüllen. Im Falle einer Infektion oder Entzündung erkennen nicht-hämatopoetische und hämatopoetische Zellen wie Immunzellen und hämatopoetische Stamm- und Vorläuferzellen das eindringende Pathogen durch Mustererkennungsrezeptoren (PRR). Toll-like Rezeptoren (TLR), eine Gruppe von PRRs, erkennen mikrobielle Produkte aus exogenen Pathogenen und induzieren eine zellabhängige Proliferation, Differenzierung, Migration sowie Proteinproduktion und Sekretion, um eine effiziente Immunantwort gegen eindringende Pathogene zu generieren . Neuste Ergebnisse haben gezeigt, dass nicht nur die peripheren reifen Immunzellen, sondern auch HSC im BM auf eine Infektion durch Erfassung von Pathogen-assoziierten molekularen Mustern (PAMPs) durch PRRs oder proinflammatorische Zytokine durch jeweilige Zytokinrezeptoren reagieren können.

Wir haben bereits gezeigt, dass eine sich wiederholende *in vivo* Stimulationen mit Lipopolysaccharide (LPS), welches eine gramnegative bakterielle Infektion durch Toll-like-Rezeptor 4 (TLR4) Stimulation rekapituliert, die Proliferation von ruhenden HSC fördert. In einem Teil meiner Dissertation, habe ich daher die Konsequenzen für die HSC-Funktion durch die LPS-Herausforderung und die zugrundeliegenden molekularen Mechanismen erforscht. Diese Studie zeigt, dass die direkte Aktivierung von TLR4 die konkurrierende Wiederbesiedlungsfähigkeit von HSC über TRIF, aber nicht MYD88-vermittelte Signalwege limitiert. Auch erhöht sich durch LPS-Stimulation die Proliferationshistorie und DNA-Schädigungs- und Reparaturantworten in HSC. Die pharmakologische *in vivo* Hemmung der reaktiven Sauerstoffspezies (ROS) und der p38 Mitogen-aktivierte Proteinkinase (MAPK), hemmt die LPS-induzierte HSC-Proliferation und Dysfunktion, und identifiziert ROS-p38 als verantwortliche TLR4-

Downstream-Signale. Diese Ergebnisse deuten darauf hin, dass eine systemische Infektion eine Akkumulation von genetischen Ereignissen in HSC verursachen könnte, die schließlich sogar zu einer bösartigen Transformation und schließlich zur Entwicklung von Leukämie führen könnten.

Die HSC Homöostase wird in einer spezialisierten Mikroumgebung im BM erzeugt, der sogenannten BM HSC Nische. Die zelluläre Eigenschaft von hämatopoetischen Stammzellen (HSC), d.h. Selbsterneuerung und Differenzierung, wird streng kontrolliert, um die lebenslange Blutproduktion über extrinsische Faktoren, die teilweise von BM-Nischenzellen geliefert werden, zu gewährleisten. Während die zellulären und molekularen Komponenten der Maus-BM-Nische ausführlich in Tieren experimentell untersucht wurden, ist wenig über die humanen BM-Nischenkomponenten bekannt.

Im zweiten Teil meiner Dissertation benutzte ich eine entwicklungstechnische Gewebe Engineering-Methode, die es ermöglicht, *ex vivo* eine menschliche Knorpel Vorlage mit menschlichen erwachsenen, BM-abgeleiteten mesenchymalen Stromazellen (MSCs) zu erzeugen und *in vivo* durch endochondrale Ossifikation in menschliche Knochenorgane, sogenannte “Ossicle”, zu entwickeln, um menschliche BM Nischenhomöostase zu studieren. *Ex vivo* entwickelte Ossicle wurden in immun-defiziente menschliche Zytokin-Knock-in-Mäuse implantiert, und mit Nabelschnurblut (CB) -abgeleiteten menschlichen CD34 + -Zellen transplantiert, um die menschliche Hämatopoese nach sublethaler Bestrahlung zu rekonstituieren. Diese Studie zeigt, dass menschliche MSC-abgeleitete Ossicle ein Maus-Gefäßnetzwerk und eine reife trabekuläre Knochen-ähnliche Struktur bilden und eine funktionelle menschliche Hämatopoese mit phänotypischen HSC entwickeln. Dies lässt vermuten, dass menschliche Ossicle als Nische zur Unterstützung der menschlichen Hämatopoese dienen können. Darüber hinaus zeigt eine erfolgreiche Rekonstruierung der humanisierten BM-Nische durch die Überexpression von SDF-1α, einem Chemokin-Protein, das für die Instandhaltung von HSCs verantwortlich ist, eine Zunahme der Erhaltung von funktionellen humanen HSC und Vorläuferzellen.

Diese Ergebnisse deuten darauf hin, dass Knochenorgane, die *in vivo* von erwachsenen BM MSC entwickelt wurden, allogene humane HSCs und Hämatopoese unterstützen können, welches die funktionalität zeigt. Die Entwicklung einer heterotrophen humanen BM-Nische, die transplantierbar und gentechnisch rekonstruierbar ist, dient als Plattform, die es ermöglicht, die Physiologie und

Pathophysiologie einer gesunden und kranken menschlichen Hämatopoese in einer optimierten Umgebung *in vivo* zu untersuchen.

INTRODUCTION

Hematopoiesis in steady state

Hematopoiesis is a lifelong dynamic process by which all cells of the blood system are produced from a small population of hematopoietic stem cells (HSCs) in a hierarchical manner. HSCs with lifelong self-renewal ability give rise to progenitor cells that differentiate into lineage-committed progenitors and ultimately produce all lineages of mature blood cells, including red blood cells, platelets, leukocytes of myeloid lineage, lymphoid lineage and dendritic cell populations[2-4] (Figure 1). As most mature cells have a limited lifespan, they have to be replenished by HSC-derived cells to maintain tissue homeostasis throughout the lifetime of an organism. In adult humans the bone marrow tissue is about 3 kg, with approximately 0.8 – 1.5 x 10^{12} bone marrow cells, releasing each day 2.2 x 10^{11} red blood cells, 0.45 – 1.2 x 10^{11} neutrophils and 1 – 1.75 x 10^{11} platelets that have to be replaced by the hematopoietic system[5-7]. Furthermore, hematopoiesis is tightly controlled by cell-intrinsic (epigenetic and transcriptional programs) and cell-extrinsic factors (growth factors, cytokines, hormones, supporting cell types in the bone marrow). Most of our understanding of physiology of hematopoiesis comes from studies in mice. To also gain mechanistic insight in human hematopoiesis the development of humanized mouse models might be of high value.

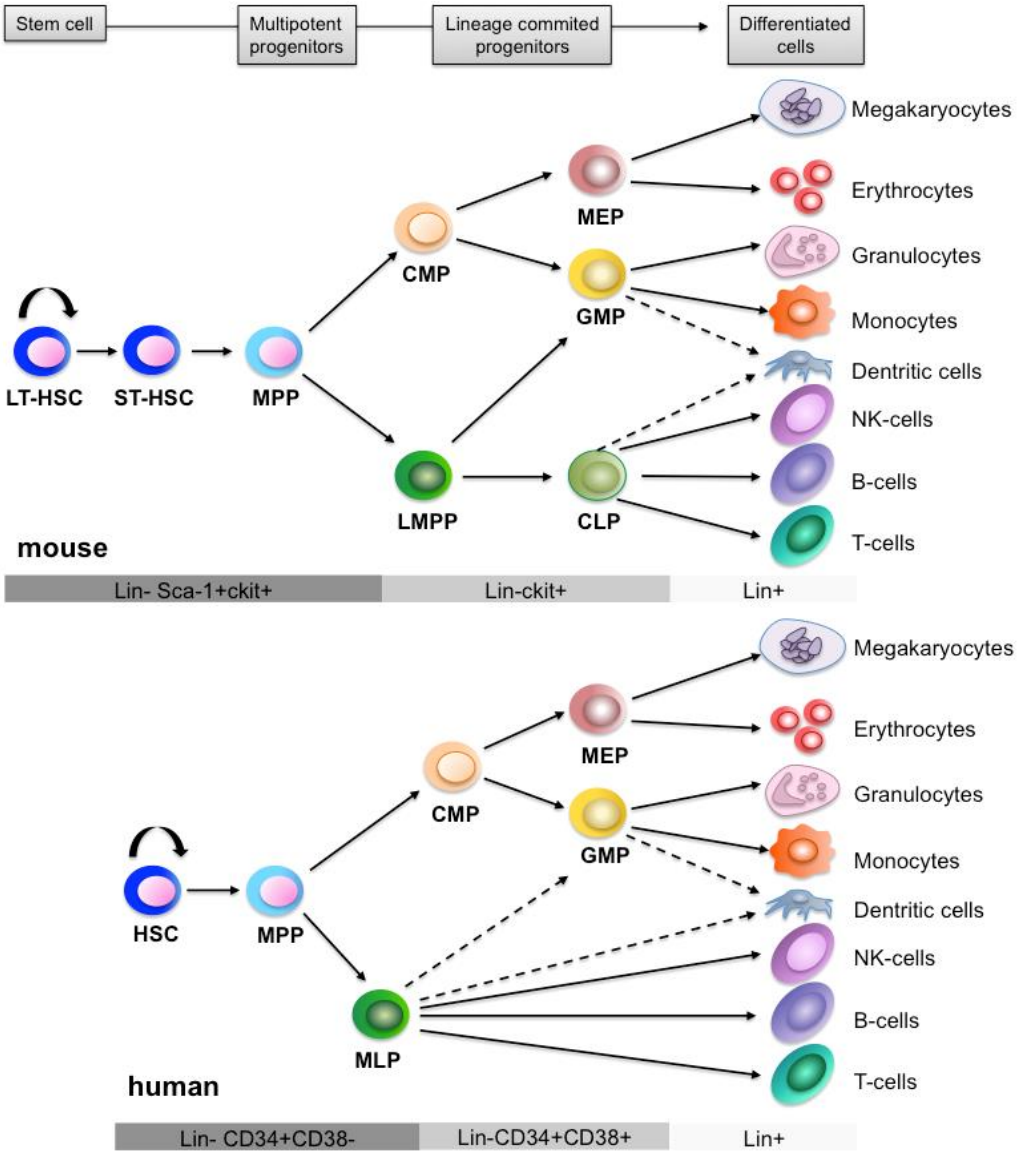

Figure 1 Hierarchy within the mouse and human hematopoietic system.
The hematopoietic system is a hierarchy maintained by self-renewing hematopoietic stem cells (HSCs). In mouse, HSCs can be subdivided based on their ability to sustain mature blood cells in long-term and short-term HSCs (LT-HSC and ST-HSC). HSCs give rise to multiple types of progenitors which have enormous proliferative potential but limited self-renewal capability, e.g. multipotent progenitors (MPP), lymphoid-primed multipotent progenitors (LMPPs, in humans: MLP), common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), granulocyte–macrophage progenitors (GMPs), megakaryocyte–erythrocyte progenitors (MEPs). Finally, on the bottom of the hierarchy are mature hematpoietic cells of different lineages that do not self-renew or have limited ability thereof and are continously replenished by upstream progenitors. NK-cells, natural killer cells. (Based on Doulatov et. al., Cell Stem Cell 2012)

Hematopoietic stem cells

The existence of cells that are characterized by loss of pluripotency but with self-renewal capacity and a differentiation ability restricted to hematopoietic tissue was first demonstrated by Till and McCulloch in 1960[8]. They showed that cells from the BM have the ability to self-renew while at the same time differentiate into multiple lineages.

In vertebrates, HSCs are generated during embryonic development. The first wave of mouse HSCs precursors of embryonic origin emerge in the yolk sac, starting from embryonic day (E) 7.5 and initiate embryonic red blood cell production. It was shown that these primitive HSCs were multipotent and capable of long-term reconstitution at E10 in the aorta-gonadmesonepros region (AGM) and the placenta[9,10]. Further development of HSCs takes place in the fetal liver (FL) through the expression of MHC class I and CD45[11]. During this stage HSCs are largely cycling and undergoing symmetric cell divisions. HSC generation is completed by E14 (so called definitive HSCs) and HSCs start to migrate to thymus, spleen, and further to the BM where they reside during adult hematopoiesis in a relatively quiescent state.

The multipotent progenitor pool is heterogeneous and can be separated into quiescent long-term self-renewing HSC (LT-HSC) with an inactive replication machinery and low metabolism; transiently active self-renewing HSC (short-term HSC, ST-HSC) with an active replication machinery and low metabolism; and non-self-renewing multipotent progenitors (MPP), with active replication machinery and active metabolism[12]. Studies indicate that most of the mature cells are derived primarily from short-term HSCs (ST-HSCs)[13]. This suggests that steady-state hematopoiesis in mouse is primarily sustained by hematopoietic progenitors (20-38% in cell cycle) rather than by the HSCs themself[13-15]. To study HSC behavior *in vivo*, it is necessary to isolate mouse HSCs from total BM cells to high purity. They can be phenotypically distinguished from more differentiated progenitors and mature cells by expression of their surface markers. In mouse, LT-HSCs are defined by their expression of Lin-Sca1+Kit+CD34-CD150+CD48-. They occur at frequencies of about 0,005% among total BM cells. In contrast, phenotypic human HSCs have been defined by the expression of Lin-CD34+CD38-CD90+CD45RA-[16]. It was shown that 0.2% of human CD34+ cells are circulating in the peripheral blood and 1,8% are present in the adult human BM[17]. Better defined HSCs (Lin-CD34+CD38-CD90+CD45RA+) have a frequency of 0.01 – 0.2 % within BM Mononuclear cells[4,18].

HSCs replenish hundreds of billions of blood cells daily while they self-renew to maintain themselves over the lifetime of the organism. The self-renewal capability of HSCs is experimentally defined by their ability to reconstitute the entire blood system upon transplantation into a lethally irradiated recipient[19]. The frequency of HSCs in mice is less than 2 per 10^8 BM cells, which means approximately 11000 – 22000 HSCs/mouse. Interestingly, in humans the estimated number of HSCs is of the same order than in mice[20]. However, this is under debate (Nombela-Arrieta and Manz, submitted).

In steady-state HSCs are maintained in a quiescent or dormant state in the BM. The cell cycle activity of HSCs over the lifetime is equally dynamic and reflects the needs of the organism at any given time (Figure 2). Only around 5-10% of total HSCs are in cell cycle (reviewed in [21]) and 90-95% of HSCs are dormant (G0 phase of cell cycle), which suggests that only a few HSC clones give rise to mature blood cells at any time[22,23,13,24].

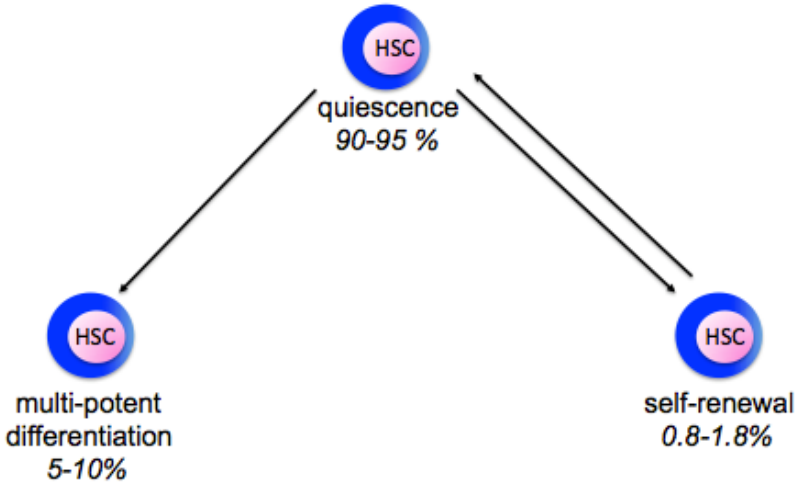

Figure 2 Dynamic activities of HSCs.
In order to preserve lifelong hematopoiesis, HSCs are mostly quiescent (90-95% of total HSCs). To maintain the stem cell pool they undergo symmetric cell divisions to self-renew, meaning HSCs produce daughter cells identical to them. At the same time 5-10% undergo asymmetric cell division to differentiate and generate more mature and specialized blood cells.

Nevertheless, it is postulated that quiescent HSCs contribute to the hematopoiesis when the proliferative capacity of the active ST-HSC clone is exhausted. Our group has shown that dormant HSCs divide only once every 39 days, while previous studies indicate that dormant HSCs divide only once every 145d or more[1,25]. These contradictions might arise from technical issues. Several cytokines like interferon

(IFN)α[26], IFNγ[27], granulocyte colony stimulating factor (G-CSF)[28] and TPO[29] were shown to induce activation of dormant HSCs. The balancing act between proliferation, self-renewal, quiescence and differentiation is a complex interplay between cell-extrinsic (i.e., transforming growth factor-β (TGF-β), Wnt, Hedgehog, and Notch) and cell-intrinsic regulatory pathways (i.e., cell cycle regulators and the PI3-kinase signaling pathway) (reviewed in[21]). A dysregulation of these processes might transform HSCs and progenitors into disease-initiating leukemic stem cells (LIC) and cause BM failure or Leukemia.

The hematopoietic niche

In 1978, R. Schofield proposed the concept of the stem cell niche[30]. He suggested that HSCs reside within a distinct threedimensional structure within a specific anatomic location in the BM, that is responsible for their regulation. Since this concept, various kinds of specific cells have been identified which regulate HSC self-renewal, differentiation and quiescence.

The bone marrow niche cells consists of hematopoietic (macrophages[31,32] and megakaryocytes[33,34]) and non-hematopoietic cells (adipocytes[35], osteoblast/osteoclast cells[36-39], endothelial cells[40 41], Schwann cells[42], as well as specialized stromal cells as for example Nestin-positive mesenchymal stromal cells (MSCs)[43], CXCL12 abundant reticular cells (CARs)[44,45], and leptin receptor-positive cells (LepR+ cells)[45]). These BM cells build the so-called BM-niche that directly or indirectly regulates HSC self-renewal, differentiation and localization. Furthermore the BM niche maintains the quiescent state of HSCs, which is essential in preserving their self-renewal capability. The HSC niche was proposed as a regulatory unit that limits the entry of HSCs into the cell cycle, thereby protecting them from exhaustion or from errors in DNA replication.

The interface of bone and bone marrow is known as the endosteum. A layer of bone-lining cells that can differentiate into bone-forming osteoblasts and bone-resorbing osteoclasts cover the endosteal surface. Arteries carry oxygen, nutrients, and hematopoietic growth factors into the bone marrow and are surrounded by Nestin+ MSCs. Sinusoids are specialized venules that form a reticular network of fenestrated vessels that allow cells to pass in and out of the circulation. Sinusoids are

often associated with megakaryocytes, reticular cells producing the chemokine CXCL12, and mesenchymal progenitors (LeptR+ MSCs).

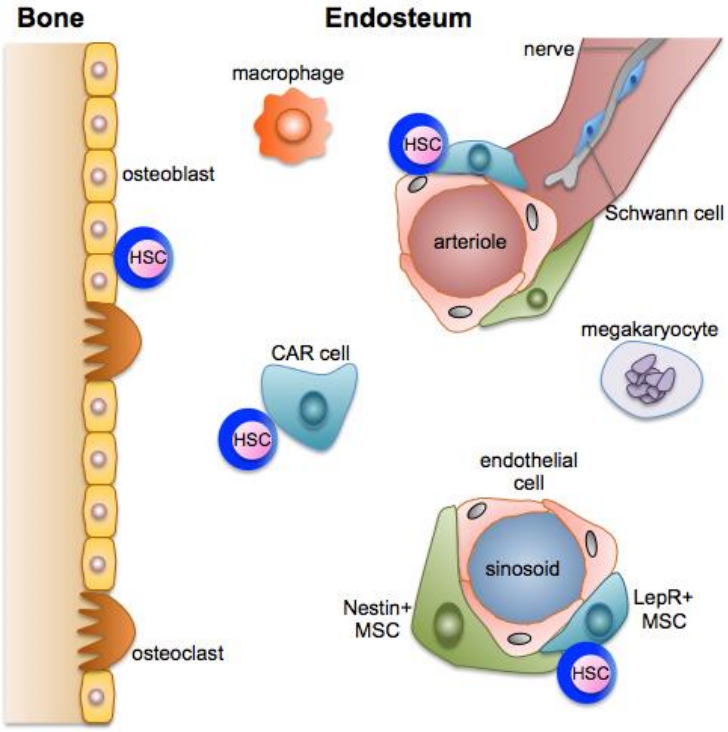

Figure 3 Illustration of the BM HSC niche.
Previously reported niche cells are depicted. The BM niche is composed of several different cell populations that contribute to the regulation of hematopoiesis. HSC, hematopoietic stem cells; CAR cell, CXCL12 abundant reticular cell; MSC, mesenchymal stromal cell; LepR, Leptin receptor.

Although the exact location of HSCs is still under debate, it is now generally accepted that adult HSCs localize primarily close to or in direct contact with the sinusoidal perivascular regions within the trabecular bone marrow cavities[46-48] and not in endosteal or osteoblastic niche. Several cellular components have been suggested to comprise the endosteal niches; these include osteoblasts, osteoclasts, macrophages, sympathetic neurons and vascular endothelial-cadherin+ sinusoidal endothelial cells (SECs)[49]. By contrast, perivascular niches are located more centrally in the BM cavities and contain endothelial cells, Nestin+ Mesenchymal stromal cells (MSC), Leptin receptor + perivascular stromal cells, Swann cells, CXC-chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells, reticular cells and megakaryocytes[50]. The BM HSC niche has been shown to express factors that are essential for HSC maintenance and localization, such as thrombopoietin (TPO),

Angiopoietin (Ang)-1, CXC-chemokine ligand 12 (CXCL12) and stem cell factor (SCF). For example, Ang-1 is expressed and secreted by osteoblasts, and interacts with Tie2, a tyrosine kinase receptor, that is expressed on quiescent LT-HSCs, to enhance the ability of HSCs to become quiescent[39].

Comparatively little is known about the cellular and molecular components of the human BM niche. Some studies showed that HSCs expand *in vitro* when co-cultured with human endothelial cells or MSCs, suggesting that human HSCs might be supported by vascular niches[51]. In contrast, immunohistochemistry of human BM indicates that human HSCs preferentially localize to the trabecular areas of the BM[52]. As in mice these areas contain Nestin+ MSCs, Endothelial cells, osteoblasts and macrophages[53,54]. Nevertheless, the knowledge gained from mouse studies cannot be translated to humans, because of the fundamental differences in immune function that exist between species. It has been noticed that altering the murine environment in favor of human hematopoiesis, by replacing mouse cytokines with their human counterparts, is beneficial to the output of human HSCs and differentiated cells. Therefore over the last decade, researchers put a lot of effort to develop humanized mice models in order to study human healthy and malignant hematopoiesis (reviewed in[55]).

Hematopoiesis under inflammatory conditions

During infection, immune cells are recruited to the local site of infection and consumed upon activation during the immune response against the invading pathogens[56,57]. Thus, the short-lived immune cells need to be subsequently replenished by upstream hematopoietic stem and progenitor cells (HSPCs) to reestablish homeostasis of the hemato-lymphoid system during and after the infection ceases. Inflammatory response protects the body from infection and injury but can itself become dysregulated.

Hematopoietic stem cell response to stress

Hematopoietic stress such as bleeding and infection can lead to demand-adopted hematopoiesis. Given the fact that HSCs are mostly in cell cycle quiescence (G0), understanding the activation and contribution of HSCs to hematopoietic homeostasis during inflammation is becoming a major focus of research in the field[3,58]. Recent findings have indicated that not only mature immune cells, but also HSPCs can

respond to infection by directly sensing pathogen-associated molecular patterns (PAMPs) through respective pattern recognition receptors (PRR)[59-61] or by detecting via respective cytokine receptors, pro-inflammatory cytokines, released by activated immune cells during infection[26,27,62]. Initiation of an inflammatory response during an infection involves, amongst others, one family of receptors, the toll-like receptors (TLRs). For example, TLR4, which senses lipopolysaccharide (LPS) and TLR2, which senses bacterial lipoproteins. Our group has previously shown that repetitive injection of LPS drives quiescent HSCs into cell cycle and increases their turn-over from 1 division within 39 days during steady-state to 2-4 divisions within 3 weeks during LPS challenge[1]. However, whether this is a direct or indirect action of LPS on TLR expressing HSCs has not been elucidated. Upon TLR stimulation HSCs are shown to secrete pro-inflammatory cytokines, e.g. IL-6, TGF-β, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) to promote myelopoiesis[63]. Since HSCs also express a broad spectrum of inflammatory cytokine/chemokine receptors they can detect pro-inflammatory signals, released by activated immune cells or the BM niche cells, in response to stress (e.g. IFN-α/γ). Also, studies show that cytokines produced by immune cells in response to viral or bacterial infection, such as IFN-α and IFN-γ can activate quiescent HSCs [26,27,62].

It has been shown that a chronic infection can impair HSC function and cause BM failure[27,64]. In humans, recent population-based epidemiological studies have demonstrated that a previous history of infectious and autoimmune disease is associated with an increased risk for acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS)[65,66]. This suggests that chronic inflammation, which induces proliferation of dormant HSCs, and thus increases their divisional history might increase accumulation of genetic alterations. In addition an SNV analysis of hematopoietic stem cells of healthy individuals from newborn until the age of 70 shows an accumulation of mutations with increasing age [67]. Surprisingly, the number of detected mutations in healthy adults were similar to the once detected in AML patients of the same age. Other studies indicate that 83% of blood-specific mutations, from healthy individuals with advanced age, were from 19 leukemia and/or lymphoma-associated genes [68]. This data suggests that self-renewing HSCs accumulate background mutations as a function of age.

Hematopoietic stem cell niche response to hematopoietic stress

It has been shown that not only hematopoietic cells express TLRs, but also endothelial cells[69,70] and MSC populations, including nestin+ MSCs and CAR cells[71] and thereby might contribute to the innate immune response (Figure 4). TLR activation of MSCs not only leads to proliferation, differentiation and tissue regeneration [72-74] of themselves but also to the regulation of proliferation and differentiation of HSPCs[75,76].

For example, *in vitro* studies show that LPS induced TLR4 activation on MSCs leads to the production of G-CSF, GM-CSF, M-CSF, IL-6 and IL-11[76]. *In vivo* studies suggest that BM endothelial cells as well as MSCs produce monocyte chemotactic protein-1 (MCP1) and promote monocyte migration[75]. Moreover, a direct LPS - TLR4 activation in endothelial cells leads to the production of G-CSF, which induces the recruitment of neutrophils to infectious sites and in parallel induces granulocyte differentiation and survival in order to replenish rapidly consumed neutrophils [70,77,78].

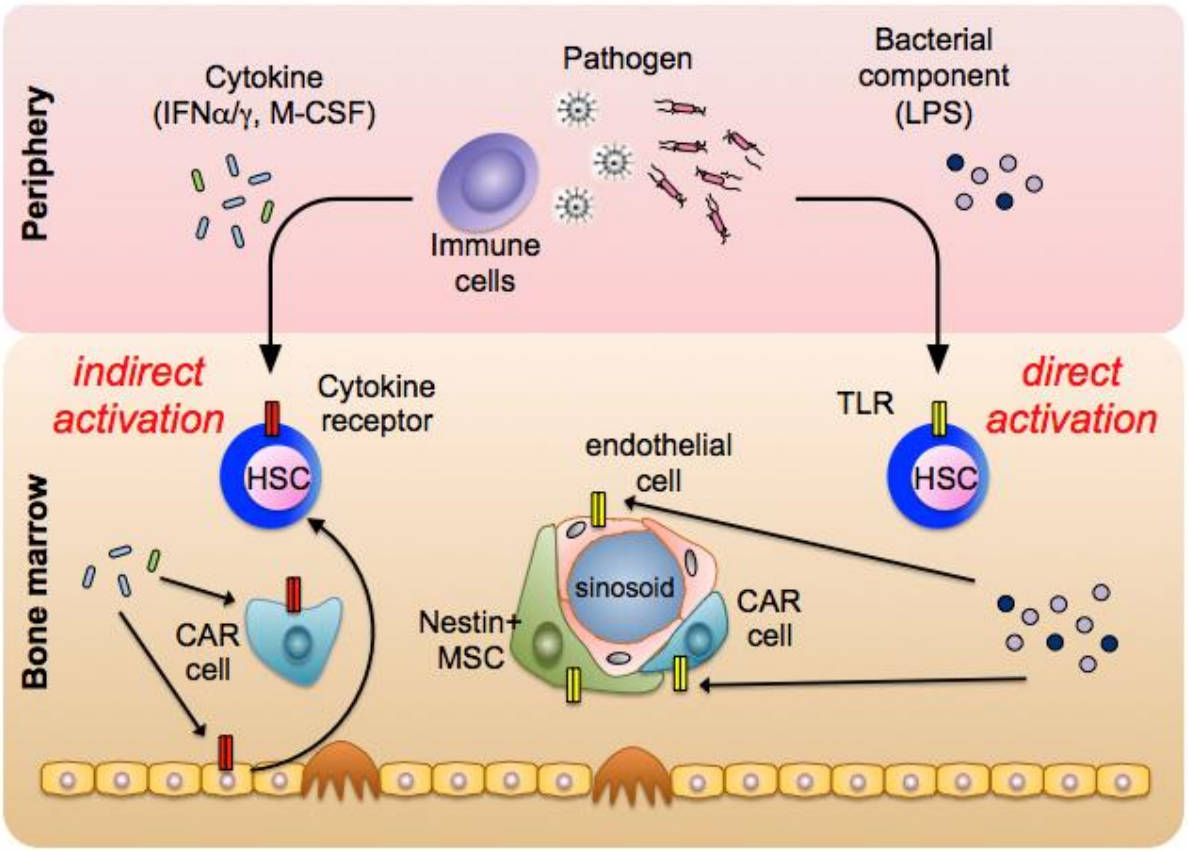

Figure 4 Illustration of the BM HSC niche during inflammatory stimulus.
Figure summarizes previously reported studies. Hematopoietic and non-hematopoietic cells are activated in direct and indirect fashion during inflammation. HSC, hematopoietic stem cells; CAR cell, CXCL12 abundant reticular cell; MSC, mesenchymal stromal cell; LPS, Lipopolysaccharid; TLR, Toll-like receptor; IFN-α/γ, interferon-alpha/gamma; M-CSF, Macrophage colony-stimulating factor.

The BM niche can be regulated by extrinsic factors, e.g. inflammatory cytokines. In detail, G-CSF, which is often induced during infection, leads to an expansion of CAR cells and suppresses mature osteoblasts[71] and CXCL12 expression in multiple stromal populations, e.g. endothelial cells[79,80]. Furthermore, Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, leads to an increased Notch activation on HSCs. Several studies suggest that IL-1 and TNF-α induce GM-CSF production in endothelial cells[81,82]. IFN-γ released from Cytotoxic T cells promotes the IL-6 production in MSCs and leads to an activation of hematopoietic progenitors[83].
Together, these results demonstrate that the BM plays an important role in hematopoietic regulation during inflammation.

Engineering of a hematopoietic niche

The BM niche cells provide survival and maintenance signals to HSCs as well as leukemia initiating cells (LICs), which can initiate, propagate, and maintain bulk leukemia [84]. A dysregulation of these signals results in abnormal differentiation and proliferation of HSCs and LICs. There is so far no good model to maintain human HSCs and LICs *in vitro*. It had been a long standing question of what cells can establish a hematopoietic microenvironment. Besides the hematopoietic system there are also other cells in the BM that can give rise to multilineage cells while retaining the ability to self-renew. Friedenstein and coworkers showed that cells from the BM can form fibroblastoid colonies (CFU-F). Furthermore these cells can be separated by their ability to adhere to plastic[85]. Later on it was found that these cells are stem cells that can differentiate into osteogenic, adipogenic, chondrogenic and myogenic mesenchymal lineages *in vitro*[86-92] and are therefore called mesenchymal stem cells. In some literature mesenchymal stem cells are also called mesenchymal stromal cells because for a long time the self-renewal and differentiation ability of these cells could be only confirmed *in vitro*. Kuznetsov et al.[93], could show for the first time that mesenchymal stem cells when implanted into immunodeficient mice, can form bone. Some Years later, it was shown that not all cells that adhere to culture plastic have self-renewal and differentiation capacity *in vivo*[94]. Therefore, they are rather restricted to be called Mesenchymal Stromal Cells (MSCs). MSCs are able to differentiate into chondrocytes through a process that is called endochondral ossification, and to form bone marrow microenvironment *in vivo*[95]. Additionally, *in vitro* maturation

(chondrogenic differentiation) of human BM-derived MSCs resulted in an accelerated formation of larger bone tissue *in vivo*[96]. Several studies have suggested that upon subcutaneous implantation of mouse or human MSCs into immune-deficient mice these cells are able to form a supportive bone marrow cavity through a vascularized cartilage intermediate which was replaced by hematopoietic tissue and bone which can attract and support murine hematopoiesis[93,97-99]. Nevertheless, there is a lack of *in vivo* models that can recapitulate the complexity of human BM environment to support normal and malignant hematopoiesis. Thus, the current xenotransplant mouse models are not able to reproduce the physiological interactions between HSCs and a human BM microenvironment as human HSCs are engrafted in a mouse BM microenvironment. A recent study has shown that when comparing MSC from different tissues such as BM, white adipose tissue (WAT), umbilical cord (UC) and skin, only BM-derived MSCs are able to form a bone marrow cavity through a vascularized cartilage intermediate[100]. Also, this human derived hematopoietic niche could home and maintain murine long-term HSCs as well as human HSCs defined as CD34+CD38-CD90+CD45RA+ after cord blood transplantation. Interestingly, the human HSCs that are maintained in the ectopic niche are functional as shown by serial transplantation[101]. Other work by Chen et al.[102] suggested a model of an ectopic human extramedullary BM that can be engrafted with human normal and leukemic cells. Furthermore, they showed the flexibility of this model by genetical manipulation through knock down of hypoxia-inducible factor 1α in MSCs, which leads to a significant reduction of human leukemic cell engraftment. However, there is no evidence that the human MSCs undergo endochondral ossification in mice, which is required for a functional niche formation[97]. In addition, Holzapfel at al.[103] developed an engineered humanized bone construct by seeding MSCs on a tubular medical-grade polycaprolactone (mPCL) scaffold followed by a culture in a rotating bioreactor before implanting it into NSG mice. By inducing differentiation of MSC *in vitro* already they were able to recapitulate morphological features and biological functions of a human HSC niche. Although they show that human HSC and progenitors engraft predominantly within the humanized bone environment, there is a lack of functional data in their studies. MSCs are not only present in the BM but also in adipose tissue, umbilical cord, placenta, amniotic fluid, dental pulp, skeletal muscle, tendons, and synovial membrane[104]. Nevertheless, Reinisch et al. could show that only MSCs from the BM are able to form a bone cavity that can maintain

human healthy and malignant cells when implanted in NSG mice[100,101]. Most of the recent studies do not or only in a small amount show the mineralization of the newly formed tissue, which does not mimic a fully regenerated bone organ. These limitations could be overcome though the establishment of a tissue engineering protocol where human adult BM-derived mesenchymal stromal cells (MSCs) are differentiated *ex vivo* into hypertrophic cartilage, and, upon *in vivo* implantation into immunodeficient mice, develop to a fully mature bone organ via endochondral ossification (Figure 5).

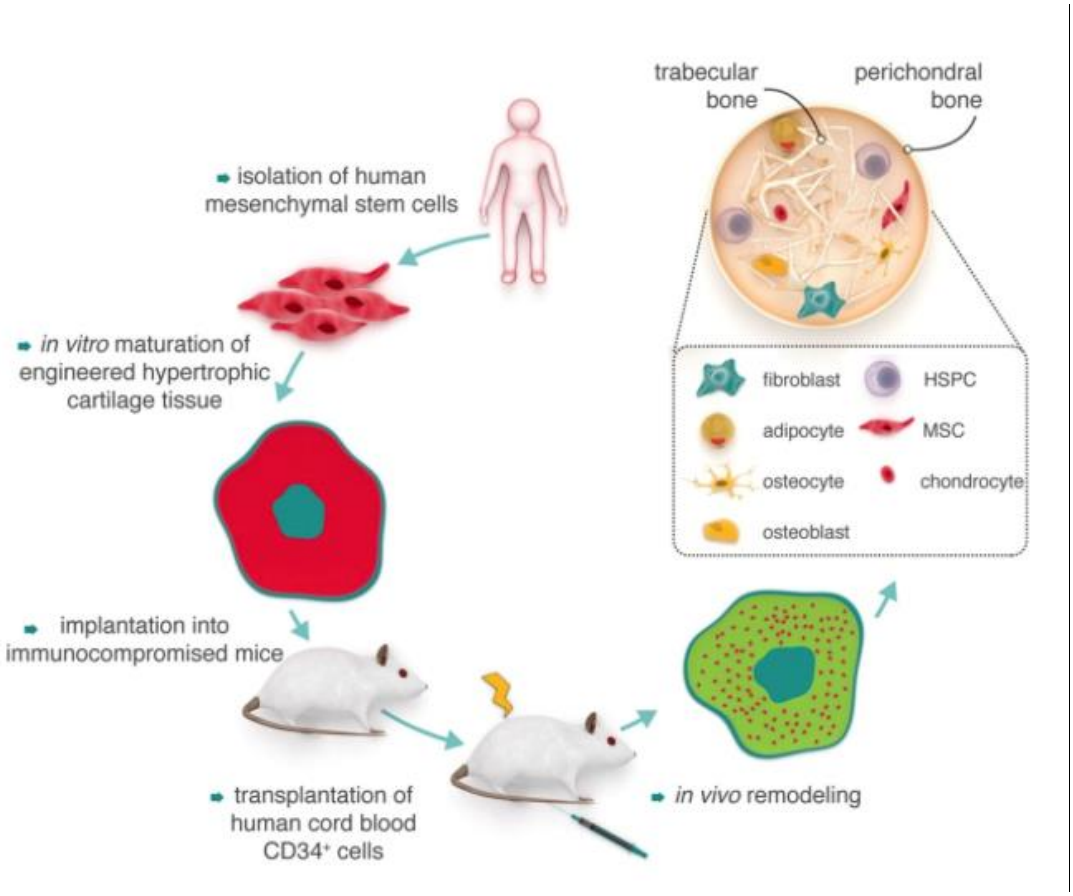

Figure 5 Engineering of heterotopic human niches in a xenograft mouse model. Illustration of *in vitro* and *in vivo* engineering of a human bone marrow niche. (From Theocharides, Rongvaux, Fritsch, Flavell and Manz, Hematologica, 2016)

AIM OF THE THESIS

HSCs and Inflammation

It remains exclusive how the primitive hematopoietic stem and progenitor cells (HSPCs) react to inflammatory-associated molecules and integrate danger signals to enhance and sustain blood production, and how these impact on HSPC function. Therefore, it is important to understand the effects of (chronic) inflammatory stimuli on HSC homeostasis and pathogenesis. These questions where addressed by setting the following aim:

Aim 1: Effect of bacterial infection on HSCs

Here we first dissected whether the action of LPS on HSCs is a direct or an indirect effect. Further, we wanted to analyze downstream TLR4 signaling and finally show the impact of chronic inflammation on the HSC function.

Modelling Human HSC Environment (Niche)

Most of the findings about the HSC niche come from mouse studies, as there is no good model to study human HSCs in their native environment. To overcome these limitations, we established a tissue engineering protocol where human adult BM-derived mesenchymal stromal cells (MSCs) are *ex vivo* differentiated into hypertrophic cartilage, and, upon *in vivo* implantation into immunodeficient mice, develop into a fully mature bone organ via endochondral ossification. With this model we addressed the following aims:

Aim 2: Engineering a human BM niche to maintain human HSC function

We generated human ossicles and tested if they sustain robust hematopoietic production and long term maintenance of human HSCs in immunodeficient animals with a human hemato-lymphoid system (HHLS).

Aim 3: Reengineering a human BM niche to study human HSC niche factors

To characterize the human HSC maintenance genes, we genetically modified human MSC using overexpression *in vitro*, we over expressed SDF-1α, a human HSC maintenance gene, in the ossicle to study the effect of the overexpression on human HSC function.

RESEARCH ARTICLE 1

(in revision)

Direct TLR4-TRIF-ROS-p38 signalling in hematopoietic stem cells induces proliferation and decreases competitive fitness

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Running title: Direct TLR4 ligation on HSCs limits their fitness
Keywords: hematopoietic stem cell, gram-negative bacteria, toll-like receptor, oxidative stress

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Summary

Bacterial infection leads to consumption of short-lived hematopoietic innate immune effector cells, which need to be replenished from hematopoietic stem and progenitor cells (HSPCs). HSPCs express various pattern recognition receptors, such as Toll-like receptors (TLRs), the ligation of which induces their mobilization, cytokine production and myeloid differentiation. However, it remains unclear how TLR activation impacts on most upstream, self-renewing HSC. Here, we show that TLR4 stimulation with lipopolysaccharide (LPS), recapitulating a systemic gram-negative bacterial infection, directly induces proliferation of dormant HSCs *in vivo*. Sustained exposure to LPS causes genotoxic stress in HSCs and impairs their competitive repopulation activity via activation of TLR4-TRIF-ROS-p38-, but not MyD88-signalling. This process can be inhibited pharmacologically without preventing emergency granulopoiesis. While direct TLR4 activation on HSCs might be beneficial to fight acute infection, our findings demonstrate that prolonged signaling has detrimental effects on HSCs, which possibly might add to chronic inflammation-associated malignant HSPCs transformation.

Highlights

- Direct TLR4 activation on HSCs induces HSC cycling
- Sustained TLR4 activation on HSC impairs their competitive repopulating ability
- TLR4 stimulation causes genotoxic stress in HSCs via ROS-p38
- Inhibition of TLR4-TRIF-ROS-p38 signalling prevents LPS-induced HSC dysfunction

Introduction

Hematopoietic homeostasis is maintained by hematopoietic stem cells (HSCs) that produce all blood cells through intermediate, subsequently lineage-committed progenitors[4]. In contrast to highly proliferating progenitors, a significant fraction of HSCs do not contribute to hematopoiesis in steady-state and are in quiescence with low metabolic activity, a presumably “safeguard” mechanism to protect HSCs from genotoxic reagents and maintain integrity of HSC function[3,13]. Self-renewal and differentiation of HSC are tightly regulated by intrinsic and extrinsic factors, and loss of this regulation leads to hematopoietic aplasia or neoplasia, respectively[105]. HSC function is preserved in a bone marrow (BM) microenvironment, referred to as “niche”, in which various type of hematopoietic and non-hematopoietic cells express vital factors for HSC homeostasis[106].

During hematopoietic stress such as infection and inflammation, where short-lived mature hematopoietic immune effector cells are activated and consumed, HSPCs in the BM need to tailor their cellular output to meet the enhanced demand of blood production. To this end, they need to integrate respective signals, ultimately stemming via various signalling cascades from the peripheral sites of inflammation. Recent findings have proposed two possible, not mutually exclusive regulatory mechanisms of HSPC response during systemic bacterial or viral infections: a.) a cytokine-mediated mechanism in which pro-inflammatory cytokines are secreted at peripheral, immune active sites and/or locally in the BM, and induce proliferation of HSPCs via their respective cytokine receptors and the downstream signalling cascades[26,27,58,62,107,108]; b.) a direct sensing mechanism, in which HSPCs themselves recognize pathogen-associated molecule patterns (PAMPs) via the respective pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and increase their proliferation, differentiation and migration[59-61,109]. Indeed, as a combination of both mechanisms, a recent study revealed that upon direct TLR activation, HSPCs are capable of producing an array of pro-inflammatory cytokines, which, likely in a paracrine, self-sustaining manner, enhance proliferation and myeloid differentiation under neutropenic conditions *in vivo*[63].

However, it remained thus far an unresolved fundamental question, if most upstream, bona fide long-term, self-renewing HSCs are able to directly sense PAMPs and, if so, what would be the consequences for HSC function. We here set out to determine this by applying *in vivo* lipopolysaccharide (LPS), a conserved

paradigmatic gram-negative bacterial pattern in context of signalling deficiencies for LPS through its cognate receptor TLR4, and the respective downstream adapter molecules MyD88 and TRIF.

Results

Systemic LPS application enhances HSCs proliferation directly via TLR4

It was shown that HSPCs (Lin$^-$c-Kit$^+$Sca-1$^+$, thereafter called LKS, or LKS CD135$^-$) express TLR4[59,63], but it remained unclear if long-term HSCs express TLR4. In agreement with a recent report[109], we found that highly enriched HSC cell populations (LKS CD34$^-$CD150$^+$) that contain long-term, multilineage repopulating cells with a 25% purity[110], uniformly express the TLR4/MD-2 complex at levels comparable to or higher than multipotent and myeloid committed hematopoietic progenitor cells (Figure S1A and S1B). Moreover, we demonstrate that mRNA of TLR4 and genes such as MYD88 (myeloid differentiation primary response 88) and TRIF (also called TICAM-1), involved in respective TLR4 downstream signaling, are higher expressed in HSC than in all other cell populations tested (Figure S1C). By applying single cell divisional tracking and subsequent evaluation of biological HSC read out, i.e., self-renewal and multi-lineage reconstitution, we previously demonstrated that dormant HSCs are recruited to proliferation upon systemic LPS injections[1]. To extend these findings, WT mice were treated for two weeks with BrdU (5-bromo-2'-deoxyuridine) to label DNA of dividing cells[111], and were subsequently i.p. injected with PBS or 35 µg LPS every other day for 2 or 4 times to determine BrdU retention or loss upon proliferation (Figure 1A). In PBS-treated animals, almost all HSC (LKS CD34$^-$CD150$^+$) in BM retained the BrdU label over the two week-chasing period, indicating their quiescence over this period of time. In contrast to this and consistent with our previous findings[1], two and four-time systemic LPS injections lead to significantly decreased BrdU retention in HSC, thus demonstrating directly or indirectly LPS-induced proliferation (Figure 1B). While multipotent progenitors (MPP, LKS CD34$^+$), LKS, and myeloid progenitors (Lin$^-$c-Kit$^+$Sca-1$^-$, thereafter called LK) proliferated already in the PBS control group, proliferation was further enhanced upon LPS application as evidenced by loss of the BrdU label (Figure 1B).

As increased proliferation renders cells more sensitive to anti-proliferative chemotherapeutic drugs, we tested if LPS-induced HSPC proliferation would lead to higher hematopoietic toxicity of otherwise sub-lethal doses of 5-FU (5-fluorouracil). Indeed, immediate LPS pre-treatment on day 1 and 3 before application of two times 150 mg/kg 5-FU, resulted in significantly higher mortality, suggesting LPS-enhanced proliferation and subsequent 5-FU mediated death of functional HSPCs (Figure 1C).

This was not observed in animals pre-treated with LPS on days -7 and -5 prior to 5-FU, indicating that LPS-induced HSPC cycling is of short duration.

Both hematopoietic and non-hematopoietic cells such as endothelial cells[78] or mesenchymal stromal cells[75] express functional TLR4[58]. To test the contribution of hematopoietic cell-expressed TLR4 to HSC proliferation, we generated mixed BM chimeric mice by reconstituting lethally irradiated TLR4 deficient (*Tlr4*$^{-/-}$) mice (CD45.1^{+}/2^{+}) with 1x10^{6} WT (CD45.1^{+}) and *Tlr4*$^{-/-}$ (CD45.2^{+}) total BM cells in a 1 to 1 ratio (WT;*Tlr4*$^{-/-}$ BM chimeric *Tlr4*$^{-/-}$ mice). Upon establishment of hematopoietic homeostasis after 2-3 months, mice were treated for two weeks with BrdU, followed by 4 time application of PBS or LPS, respectively (Figure 1D). Within the same PBS-treated recipients WT and *Tlr4*$^{-/-}$ HSCs retained BrdU similarly. In contrast, proliferation was enhanced upon LPS treatment in both, WT and *Tlr4*$^{-/-}$ HSCs in the same animal, however, WT HSCs proliferated significantly more (Figure 1E and 1F). Thus, while some proliferation depends on hematopoietic cell secreted factors from WT hematopoiesis in these chimeric animals (the delta between proliferation of TLR4$^{-/-}$ HSCs upon PBS and LPS treatment), a significant, about equally sized part of proliferation is driven by direct TLR4 stimulation of HSCs (the delta between proliferation of WT and TLR4$^{-/-}$ HSCs upon LPS treatment). In order to further test the TLR4-mediated direct HSC activation, we employed CFSE (Carboxyfluorescein diacetate succinimidyl ester)-based high sensitive HSC divisional tracking[1]. Highly purified WT HSCs (LKS CD34^{-}CD48^{-}CD41^{-}CD150^{+}) or MPPs (LKS CD34^{+}) were labeled with CFSE and transferred into non-irradiated steady state *Tlr4*$^{-/-}$ recipients. Mice were i.p. treated with PBS or LPS twice or trice starting at 2 days after the transfer and analyzed at day 6 to determine divisional history of donor-derived HSC or MPPs based on CFSE dilution (Figure 1G). Almost all of transferred HSCs were found quiescent in the non- or 1x divided fraction of PBS-treated animals 6 days after initial transfer. In contrast, LPS injections significantly decreased the relative percentage of 0-1x divided cells and consecutively increased the percentage of 2-5x divided cells, indicating direct, LPS-induced HSC proliferation (Figure 1H and 1I). A similar effect occurred also in MPPs although less pronounced.

Collectively, we conclude that highly purified HSCs can be activated from dormancy to cell cycle via direct TLR4 stimulation *in vivo* and that in settings of systemic LPS spread, HSC population divisional activation occurs with about equal

strength through direct TLR4-activation on HSCs and indirect, non-hematopoietic cell-mediated pathways.

LPS challenge impairs competitive repopulating and self-renewing capacity of HSC through direct TLR4 activation

Previous studies have shown that chronic exposure of HSC to LPS damages HSC function as defined by decreased competitive transplantation and hematopoietic reconstitution[112,113,109]. However, it remained unclear whether LPS-induced HSC dysfunction is mediated by TLR4 expression on non-hematopoietic or hematopoietic cells, or both. To dissect this, we employed *Tlr4*$^{-/-}$ and WT mixed BM chimeric mice (Figure 2A). Three months after transplantation, chimeric mice were i.p. injected with PBS or LPS (35 µg) every other day 8 times over two weeks, respectively. The relative chimerism of donor-type cells (ratio of *Tlr4*$^{-/-}$- to WT-derived cells) in three major blood lineages (CD19^{+} B cells, CD3▪$^{+}$ T cells and Mac-1^{+}Gr-1^{+} granulocytes) in peripheral blood (PB) was tested monthly for up to 5 months after the last injection. Chimerism of *Tlr4*$^{-/-}$- and WT-derived cells was equal in all the lineages before the injections, resulting in a ratio of 1, and remained unchanged over time upon PBS injections as expected (Figure 2B and 2C). In contrast, a significant increase in the relative chimerism of *Tlr4*$^{-/-}$ cells over WT cells was detected in LPS injected animals, indicating that WT cells were outcompeted by *Tlr4*$^{-/-}$ cells. Terminal BM analysis revealed that, similar as in mature blood cells, hematopoietic stem and progenitor cell populations (HSC (LKS CD34^{-}), MPP (LKS CD34^{+}), CMP/GMP (LK CD34^{+}), and MEP (LK CD34^{-})) in BM were dominated by *Tlr4*$^{-/-}$ cells (Figure 2D), indicating that hematopoietic dominance of *Tlr4*$^{-/-}$ mature cells was due to higher proliferative activity of most upstream but not downstream cell populations. As both WT and *Tlr4*$^{-/-}$ hematopoiesis coexisted in the same mice and thus were exposed to the same environment, these data demonstrate that LPS activates TLR4 directly on WT cells and impairs their competitive repopulating ability, leading to dominance of *Tlr4*$^{-/-}$ hematopoiesis.

To test if the LPS-induced competitive repopulating disadvantage of WT cells resulted from a functional defect in self-renewing HSC, we transplanted 1-2x10^{6} total BM cells of primary recipients into lethally irradiated secondary recipients. Strikingly, we found that the same effect was sustained in PB and BM of secondary recipients

over 4 months, the longest time of follow-up (Figure 2E and 2F). Thus, the LPS-induced deficiency indeed affected HSCs.

To further evaluate if direct TLR4 activation on hematopoietic cells is sufficient for the observed findings with an alternative method, we generated WT;*Tlr4*$^{-/-}$ BM chimeric mice on a *Tlr4*$^{-/-}$ background, and subsequently challenged these with PBS or LPS. The competitive disadvantage of WT cells compared to *Tlr4*$^{-/-}$ cells upon *in vivo* LPS treatment was even more pronounced in the *Tlr4*$^{-/-}$ compared to WT recipients (Figure 2G and H), again documenting the consequence of direct TLR4 ligation on HSPC and their subsequent relative competitive deficiency.

As infection or LPS challenge is known to mobilize HSPC into the circulation, in part via up-regulation of endothelial cell-produced G-CSF[78,107], the observed LPS-TLR4-induced competitive HSC dysfunction might be due to differential mobilization and homing of WT versus *Tlr4*$^{-/-}$ HSC, resulting in the out-competition of WT HSCs in the BM. However, we were not able to detect differential mobilization of WT versus *Tlr4*$^{-/-}$ HSPCs in chimeric animals (data not shown). If a competitive re-homing disadvantage after LPS challenge would account for the observed out-competition, HSPC potential should be conserved in a non-competitive setting in WT cells. To test this, we injected PBS or LPS into WT mice, and isolated 3,000 LKS from BM 4 weeks after the last treatment when LPS-mobilized HSCs had migrated back to BM, as evidenced by a normal HSPC profile in BM and spleen (data not shown). The isolated LKS cells (CD45.2^{+}) were then co-transplanted with an equal number of 3,000 competitor LKS cells (CD45.1^{+}) to lethally irradiated WT mice (CD45.1/2^{+}) (Figure S2A). Subsequent monthly PB and terminal BM analysis showed that WT LKS isolated from LPS-pretreated mice harbored significantly reduced competitive repopulation capacity compared to WT LKS from PBS-pretreated mice (Figure S2B and S2C), demonstrating that LPS challenge damages HSC function, irrespective of possible migration effects in chimeric mice.

Taken together, these data directly demonstrate that *in vivo* repetitive TLR4 activation on HSCs impinges on their competitive self-renewing and repopulating ability.

LPS-induced functional HSC impairment is dependent on TRIF- but not Myd88-mediated signaling

In mature innate immune cells such as macrophages or dendritic cells, TLR4 ligation activates two distinct signaling cascades, mediated by two adaptor proteins carrying TIR (Toll-Interleukin receptor) domains, MYD88 (myeloid differentiation primary response 88) and TRIF (also called TICAM-1, toll-like receptor adaptor molecule 1), that both lead amongst others to NFκB activation at an early and later phase, respectively[114]. However, it is not known which TLR4-downstream signaling cascades are active in HSCs. We therefore investigated if the two adaptor molecules MYD88 and TRIF are involved in the LPS-induced HSC dysfunction. As acute inflammation was shown to enhance Sca-1 expression on HSPC[26], we tested if LPS challenge up-regulated Sca-1 expression, and if genetic deletion of either *Myd88* or *Trif* might abrogate it. A single LPS injection (35 µg) into WT mice induced strong Sca-1 up-regulation in BM Lin$^-$ cells 16 hours post treatment, resulting in an increase in the absolute number of phenotypic LKS and HSC (LKS CD34$^-$CD150$^+$) (Figure 3A-C). Surprisingly, *Trif*$^{-/-}$ mice, but not *Myd88*$^{-/-}$ mice, failed to increase Sca-1 expression on HSPCs, similar as observed in *Tlr4*$^{-/-}$ mice, thus indicating downstream TLR4 signaling via TRIF but not MyD88 in HSPCs. Consistent with this phenotypic observation, WT mice, reconstituted with WT and *Myd88*$^{-/-}$ or *Trif*$^{-/-}$ cells resulting in hematopoietic chimeras, and subsequently treated with PBS or LPS (Figure 3D), showed a competitive repopulation advantage for *Trif*$^{-/-}$ but not *Myd88*$^{-/-}$ cells over WT cells (Figure 3E and 3F). These data demonstrate that the functional defect of HSCs following direct TLR4 activation is mediated via TRIF- but not MYD88-signaling pathways.

Pharmacological inhibition of ROS and p38 activation prevents LPS-TLR4-TRIF-induced HSC dysfunction

To further characterize the downstream signaling cascade responsible for LPS-TLR4-TRIF mediated HSC alteration, we tested selective small inhibitors of TLR4 downstream signaling (Figure S3A). WT mice were treated with the respective inhibitor 24 and 5 hours before LPS injection (Figure S3B). BM analysis 16 hours post LPS injection showed that the phenotypic expansion of HSCs (LKS CD34$^-$ CD150$^+$) upon LPS treatment was significantly suppressed by pretreatment with inhibitors against reactive oxygen species (ROS), NAC (N-acetyl-L-cysteine), and against p38 MAPK (mitogen-activated protein kinase), SB203580 or SB202190 (thereafter called SB)[115], but not by those against ERK1/2 (MAPK 1 and 2) and

against NFκB (nuclear factor of kappa light polypeptide gene enhancer in B cells), NaS (sodium salicylate)[116] or NBD peptide (a cell permeable NEMO-binding domain peptide)[117] (Figure S3C and S3D). The most significant suppression of phenotypic HSC expansion was achieved when mice were treated with both ROS and p38 inhibitors (NAC/SB). Consistent with these findings and observations made in innate immune cells[118], flow-cytometric analysis confirmed intracellular ROS production and p38 activation as early as 2 hours after *in vivo* LPS application in HSCs (LKS $CD34^-$) and MPPs (LKS $CD34^+$) but not in CMP/GMP (LK $CD34^+$) (Figure S3E).

We addressed if ROS/p38 inhibitors can block LPS-induced proliferation of HSCs. WT;*Tlr4*$^{-/-}$ BM chimeric *Tlr4*$^{-/-}$ mice were treated with BrdU for 2 weeks and subsequently with NAC and SB at 24 and 5 hours prior to PBS or LPS injection for 4 cycles (Figure 4A). BM analysis one week after the final injection revealed that combined inhibitor treatment abolished the LPS-induced increase in proliferation of both, WT and *Tlr4*$^{-/-}$ HSC (Figure 4B).

We next addressed the question if inhibition of the ROS-p38 mediated signaling pathway can prevent the LPS-induced competitive disadvantage of HSCs. WT;*Tlr4*$^{-/-}$ BM hematopoietic chimeric mice were treated with 8 cycles of sequential inhibitors and LPS applications, and the relative contribution of *Tlr4*$^{-/-}$ over WT cells to hematopoietic chimerism in PB and BM was determined (Figure 4C). Strikingly, the combined NAC/SB treatment prior to LPS completely abolished the competitive disadvantage of WT hematopoiesis in PB (Figure 4D). The same effect was observed in HSCs, LKS and LK in BM upon termination of the experiment (Figure 4E).

Together these data demonstrate that activation of ROS and p38 through TLR4-ligation is a key step to cause HSC dysfunction and that this dysfunction can be prevented by respective pharmacological blockade.

Emergency granulopoiesis depends on TLR4-MYD88 but not TRIF-ROS-p38 signaling

We recently demonstrated that systemic LPS- or bacterial infection-driven granulopoiesis, i.e. emergency granulopoiesis, is mediated by TLR4-MYD88 signaling in endothelial cells and subsequent G-CSF release[78,108]. Given our findings on TLR4-TRIF but not TLR4-MyD88 mediated signaling in HSCs, we determined if TRIF-deficiency would affect LPS-induced emergency granulopoiesis. As expected and previously shown, *in vivo* LPS challenge of *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice resulted in

absence of the enhanced granulopoiesis, whereas *Trif*$^{-/-}$ mice showed an intact emergency granulopoietic response (Figure S4A-C). Moreover, none of the inhibitors tested had a suppressive effect on LPS-induced emergency granulopoiesis (Figure S4D and S4E).

Together these data demonstrate that immediate emergency granulopoiesis and HSC activation via TLR4 are differentially regulated by Myd88- and TRIF-dependent signals, respectively.

TLR4 stimulation induces genotoxic stress in HSCs via ROS-p38 signaling

Increased levels of ROS are associated with cell cycle induction[64] and attenuated HSC function during serial transplantation[115]. Also, ROS can cause DNA breaks and chromosomal aberrations in BM cells of a Fanconi anemia mouse model[119]. We thus tested if TLR4-mediated ROS production leads to genotoxic stress in HSCs. Mice were treated with PBS or LPS for 8 times, and HSCs (LKS $CD34^{-}CD150^{+}$ or LKS $CD34^{-}CD48^{-}CD41^{-}CD150^{+}$) and myeloid progenitors (LK or LK $CD34^{-}CD48^{-}CD41^{-}$) in BM were analyzed at different time-points after the last injection (Figure 5A). Cells were stained with antibodies against phosphorylated H2AX (γH2AX), a surrogate marker for DNA damage response[120,121], and $\gamma H2AX^{+}$ foci formation was visualized and quantified. The proportion of cells with more than three $\gamma H2AX^{+}$ foci was significantly increased in both HSCs and their progeny upon LPS treatment, and was comparable to that observed in mice that were gamma-irradiated (2Gy) two hours before analysis (Figure 5B and 5C). Surprisingly, LPS-induced $\gamma H2AX^{+}$ foci were maintained for longer than 14 days *in vivo*, indicating sustained genotoxic stress upon LPS challenge.

As it was recently shown that $\gamma H2AX^{+}$ foci formation results from replication defects in cycling HSCs rather than from DNA double-strand breaks[122], we analyzed if $\gamma H2AX^{+}$ foci correlate with cell cycle induction in HSCs. Snap-shot cell cycle analysis showed that a higher fraction of HSCs (LKS $CD34^{-}CD150^{+}$) were found in G1 at day 3 post LPS injection, but regained normal cell cycle distribution at day 14 (Figure 5D and 5E), while there was no significant difference in actively cycling myeloid progenitors (LK), consistent with BrdU labeling and dilution experiments in Figure 1B. The apoptotic rate was similar in PBS- and LPS-stimulated HSCs at any time point analyzed (data not shown). We then addressed if ROS/p38 inhibitors can block $\gamma H2AX^{+}$ foci formation. When WT mice were treated with NAC and SB prior to

LPS injections for 8 cycles (Figure 5F), the percentage of HSCs and their progeny carrying γH2AX^{+} foci at day 3 post treatment was significantly suppressed (Figure 5G), indicating that the foci formation is mediated by the ROS-p38 pathway.

Taken together, the increase of ROS levels and p38 activation upon LPS challenge leads to proliferation and causes sustained genotoxic stress in HSCs that can be blocked by ROS-p38 inactivation.

Discussion

In this study, we provide for the first time definitive *in vivo* evidence that i) quiescent HSCs in the BM sense systemically spread LPS directly via HSC expressed TLR4 and subsequently increase cycling, that ii) TLR4-stimulation on HSCs impairs their competitive repopulation ability and causes genotoxic stress via TRIF-mediated, but not MyD88-mediated, ROS-p38 signaling, and that iii) pharmacologic inhibition of TLR4-TRIF-ROS-p38 signaling prevents LPS-induced HSC dysfunction.

Although not directly addressed in our study, one might speculate that LPS (and possibly other PAMP) sensing by HSCs and subsequent proliferation might enhance hematopoietic cell production in situations of need, and thus has been advantageous and selected in evolution. However, by employing BM chimeric mice with one half of hematopoietic cells expressing TLR4 and the other half not expressing it, our study clearly demonstrates that *Tlr4*$^{-/-}$ HSCs are also induced to divide upon LPS injection, a process that must be driven by secondary, extrinsic signals such as pro-inflammatory cytokines secreted from TLR4-expressing non-hematopoietic (in the case of a TLR4 WT environment) or hematopoietic cells (in the case of a hematopoietic WT;*Tlr4*$^{-/-}$ chimera in a TLR4$^{-/-}$ non-hematopoietic environment). One potential cytokine involved in this process could be IL-6, which has been shown to be produced by HSPCs upon TLR4 activation[63] and non-hematopoietic BM cells via IFN-γ triggering upon viral infections[83]. As the relative contribution to HSC cycling by direct, HSC expressed TLR4-ligation and indirect, non-HSC expressed TLR4 ligation was of about equal strength in the experimental settings in our study, it will be challenging to dissect in which *in vivo* infectious disease situation the direct TLR4-mediated response will provide a distinct advantage to hematopoietic regulation and subsequent host defense and survival.

The observed negative consequence of direct LPS-TLR4-mediated HSC activation is a compromised competitive capacity of HSCs, defined by both

decreased hematopoietic output in competition with control-stimulated HSCs upon transplantation, as well as in a relative loss of TLR4-stimulated HSC numbers compared to TLR4-signaling deficient HSC controls. These findings are in line with the observation that LPS application over one month resulted in reduced lymphoid repopulation from HSCs in competitive conditions[113].

By dissecting TLR4 downstream signaling in HSCs, we found that HSC signal via the TLR4-associated TIR adaptor protein TRIF, but not MYD88. TLR4-TRIF triggers a downstream signaling cascade via ROS-p38, which causes cell cycle progression in HSC and leads to a genotoxic stress response that is marked by the phosphorylation of H2AX, previously reported as p38 target[123]. We here demonstrate that both genetic and pharmacological inactivation of this pathway can block HSC proliferation and dysfunction in response to LPS. Indeed, it has been reported that activation of the ROS-p38 axis upon serial transplantation results in decreased quiescence and impaired repopulating ability of HSCs[115]. In addition, we here demonstrate that pretreatment of HSCs with ROS-p38 inhibitors prior to LPS challenge maintained HSC function. This is in line with the findings that inhibition of ROS-p38 sustains HSC function *in vivo* [109,124,125] and also supports *ex vivo* expansion of both mouse and human HSCs[126,127]. On the contrary, dysregulation of ROS-p38 signaling was proposed to be a hallmark of stem cell ageing[128]. Moreover, it was shown that scavenging ROS restored the quiescence and regenerative potential of different somatic stem cells[115,124,129], and blockage of ageing-associated p38 activation ameliorated the functional decline in aged muscle stem cells[130]. How ROS-p38 activation causes HSC proliferation and dysfunction, genotoxic stress and the associated $H2AX^{+}$ foci formation, as well as possible subsequent genetic alterations is still controversial, with several studies demonstrating varying results[122,131,132]. Thus, we do not know from the data presented here if the observed enhanced cycling and genotoxic stress results in functional HSC attrition[64] or if, at least in some cases, also results in genetic lesions in HSCs. However, it is of interest that population-based epidemiological studies showed a significant positive correlation of infectious and inflammatory states and the incidence of HSPC diseases, such as acute myeloid leukemia, myelodysplastic syndromes, and myeloproliferative neoplasms[65]. Furthermore, a recent study also has shown that TLR4-TRAF6 signaling enhanced by deficiency of TIFAB (TRAF-interacting protein with forkhead-associated domain B), a haplo-insufficient gene in del(5q) myelodysplastic syndrome, causes inefficient

hematopoiesis[125]. Thus, if direct stimulation of PAMPs on HSPCs can indeed enhance acquisition of genetic events that lead to hematopoietic deficiency, clonal dominance, and finally malignant hematopoietic disease, need to be determined in future studies.

TLR4 stimulation induces progenitor cell division and differentiation to myeloid cells via MYD88[59,60] and TLR4 stimulation also induces MYD88 dependent G-CSF secretion from endothelial cells, leading to subsequent emergency granulopoiesis[78]. In contrast, we show here that TLR4 stimulation in bona fide HSCs leads to TRIF-, but not the MYD88-dependant signaling, revealing differential use of TLR4-downstream pathways in different cells of the hematopoietic hierarchy. We thus tested if ablation or inhibiting TRIF-mediated signaling would negatively affect emergency granulopoiesis. Interestingly, this was not the case. Therefore, it might be feasible to prevent HSC from LPS-induced functional impairment by pharmacologic TRIF-dependent signaling pathway inhibition, while at the same time preserving indirect HSC cycling induction as well as emergency granulopoiesis in order to augment innate immunity against systemically spread, life-threatening gram-negative infection.

In summary, our findings highlight a direct pathogen sensing mechanism in self-renewing HSCs that leads to HSC proliferation but decreases their regenerative capacity. Intervention into the TRIF-ROS-p38 signaling axis has the potential to prevent HSC dysfunction, likely without inhibition of short-term protective responses against pathogen insults. If inhibition of this pathway as means of preemptive medicine will also allow to retard ageing-associated HSC defects and ageing-associated malignant transformation, will need to be determined in future studies.

Experimental Procedures

Mice

Tlr4$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$ mice (CD45.2^{+}) were kindly provided by Dr. Shizuo Akira (Osaka University). C57BL/6 (CD45.2^{+}) and B6.SJL (CD45.1^{+}) mice were obtained from Jackson Laboratories.

Generation of BM chimeric mice, LPS challenge and serial transplantation

WT or *Tlr4*$^{-/-}$ mice (CD45.1/2^{+}) were lethally irradiated with 9.5Gy and i.v. transplanted with equal numbers (1:1 ratio) of 1x10^{6} total BM or 3,000 Lin^{-}c-Kit^{+}Sca-1^{+} cells from WT and *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, or *Myd88*$^{-/-}$ animals. Two to three months after reconstitution, BM chimeric mice were i.p. injected with 35-50 µg LPS corresponding to 35-50 EU. In serial transplantation experiments, 1-2x10^{6} total BM cells from chimeric mice were serially transplanted into lethally irradiated WT (CD45.1/2^{+}) mice.

FACS analysis and sorting

Donor chimerism in peripheral blood, BM and spleen were analyzed on a FACS Canto II. For snap-shot cell cycle analysis, total BM cells were stained with antibodies against cell surface antigens, Ki67 (B56) antibody and Hoechst33342 for DNA. For TLR4 expression, total BM cells were incubated with an antibody against TLR4/MD-2 complex together with early hematopoietic cell markers. LKS cells, MPPs or HSCs were sorted on a FACS Aria III (BD Biosciences).

Quantitative RT-PCR

HSC and HPC from BM, or mature cell populations from BM and spleen were sorted directly into lysis buffer and subjected to RNA isolation, cDNA synthesis and Real Time PCR with primers specific to the following genes: *Tlr4*, *Trif*, *Myd88*, *Mpl* and *Actb*. Relative mRNA expression of each gene was normalized against relative expression of beta-actin.

Kaplan-Meier survival following 5FU administration

WT mice were i.p. pretreated with PBS or 35 µg LPS, 1 and 3 days or 5 and 7 days before two i.p. injections of 5-FU (150 mg/kg) in a weekly interval. The survival of animals was followed over one month.

In vivo inhibitor treatment

BM chimeric mice were i.p. injected with 100 mg/kg NAC and 15 mg/kg SB203580 24 and 5 hours before PBS or LPS treatment, respectively.

BrdU labeling and retention

WT or mixed BM chimeric mice were i.p. injected with 9 mg/kg BrdU and subsequently put on drinking water containing 800 µg/ml BrdU and 5% sucrose for two weeks followed by normal water for another two weeks until analysis. One week after final PBS or LPS injections, BM cells were harvested and stained with antibodies against cell surface antigens and BrdU.

CFSE labeling and chasing

HSCs (3,000-7,835 LKS $CD34^{-}CD48^{-}CD41^{-}CD150^{+}$ cells) or MPPs (113,250 LKS $CD34^{+}$ cells) isolated from WT mice ($CD45.2^{+}$) were labeled with CFSE as previously described (Takizawa JEM 2011) and i.v. transferred into *Tlr4*$^{-/-}$ mice ($CD45.1^{+}$). Two or three days later, mice were treated with PBS or 100 µg LPS for 2 or 3 times and were analyzed on day 6 to determine CFSE dilution in HSCs and MPPs.

Immunocytochemistry

HSCs (LKS $CD34^{-}CD48^{-}CD41^{-}CD150^{+}$) and LK $CD34^{-}CD48^{-}CD41^{-}$ cells were sorted, fixed with 4% PFA for 10 min and permeabilized with 0.5% Triton X-100. γH2AX foci and nuclei were visualized with anti-phospho-histone H2AX antibody and DAPI, respectively, and images were taken on a Leica SP5 with a 63x objective lens (Leica).

Statistical analysis

All data are shown as the mean ± s.e.m., unless indicated otherwise. Kaplan-Meier survival was analyzed by Log-rank test on GraphPad Prism 5 (GraphPad software, Inc). All statistical comparisons were evaluated with Student's t-Test. ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (two-tailed t-test).

Supplemental Information

Supplemental Information for this article includes four figures and Supplemental Experimental Procedures and can be found with this article online.

Author Contributions

H.T. designed and performed experiments, analyzed data, and wrote the manuscript. K.F., Y.S., L.V.K., C.Y., K.J., and A.K.A. performed experiments. M.L. designed and analyzed data. M.G.M. designed and supervised research and wrote the manuscript.

Acknowledgment

We would like to thank S. Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University) for kindly providing *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$ mice. We also thank the Institute of Research in Biomedicine and University Hospital Zurich for the logistical and technical assistance, and the flow cytometry and microscopy core facility at University of Zurich. This work was supported by Kumamoto University International Collaborative Research Group for Life Sciences to H.T., the Swiss National Science Foundation (31003A_146924/1) to M.L., and the Swiss National Science Foundation (310030_146528/1), the Promedica Foundation (Chur, Switzerland), and the Clinical Research Priority Program of the University of Zurich to M.G.M.

Figure legends

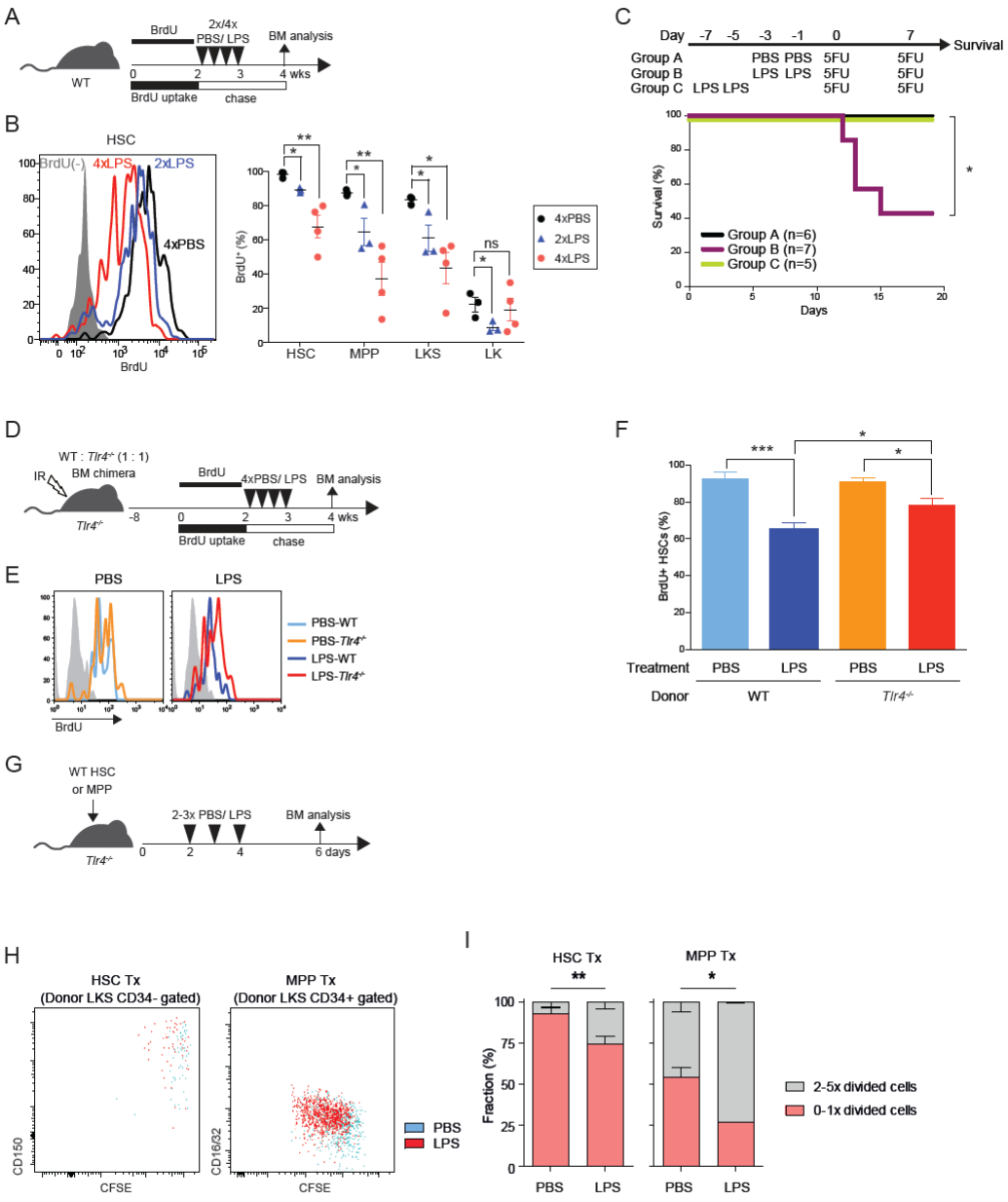


Figure 1. LPS increases proliferation of HSCs via direct TLR4 activation. (A) Experimental scheme of the BrdU label retention assay for results depicted in (B): WT mice were treated with 9 mg/kg BrdU i.p. once and fed with 800 µg/mL BrdU containing water for 2 weeks. Mice were then treated with PBS or LPS injections 2 or 4 times and BrdU label retention was determined in BM populations 1 week after the last injection. (B) Representative histogram of BrdU label in HSC ($LKSCD34^{-}CD150^{+}$) from animals injected with 4xPBS, 2xLPS, and 4xLPS. Right panel depicts percentage of $BrdU^{+}$ HSCs ($LKSCD34^{-}CD150^{+}$), MPPs ($LKSCD34^{+}$), LKS and LK

cells (4xPBS: n=3; 2xLPS: n=3; 4xLPS: n=4). Each dot represents data from individual animals. (C) Survival of mice treated as indicated with PBS (Group A: day -3 and -1; n=6) or LPS (35 µg) injections (Group B: day -3 and -1; n=7; Group C: day -7 and -5; n=5) followed by i.p. application of 150 mg/kg 5-FU at day 0 and 7. (D) Experimental scheme of BrdU label retention assay for results depicted in (E) and (F): *Tlr4*$^{-/-}$ mice were reconstituted with WT and *Tlr4*$^{-/-}$ BM in a 1:1 ratio (mixed WT;*Tlr4*$^{-/-}$ BM hematopoietic chimeric *Tlr4*$^{-/-}$ mice). Eight weeks after reconstitution, the mixed BM chimeric mice were given BrdU containing water for 2 weeks and injected with PBS or LPS (50 µg) 4 times followed by BM analysis 1 week post the last injection. (E) Representative histogram of BrdU label in WT and *Tlr4*$^{-/-}$ HSC (LKSCD34^{-}CD150^{+}) from PBS- or LPS-treated mixed WT;*Tlr4*$^{-/-}$ BM hematopoietic chimeric *Tlr4*$^{-/-}$ mice. (F) Percentage of BrdU^{+} HSCs derived from WT or *Tlr4*$^{-/-}$ donors in mixed BM chimeric *Tlr4*$^{-/-}$ mice upon PBS or LPS injections (PBS, n=7; LPS, n=8). (G) Experimental scheme of CFSE based cell divisional tracking. *Tlr4*$^{-/-}$ mice were transplanted with CFSE-labeled WT HSCs (LKS CD34^{-}CD48^{-}CD150^{+}) or MPPs (LKS CD34^{+}), and i.p. injected 2 or 3 times with PBS or 100 µg LPS followed by BM analysis at day 6. (H) Representative FACS plots of CFSE dilution versus the respective marker indicated, in donor-derived HSC (LKS CD34^{-}) or MPP (LKS CD34^{+}) from PBS (blue)- or LPS (red)-treated mice that had been previously transplanted with HSC (left) or MPP (right). (I) Percentage of 0-1x divided cells and proliferating (2-5x divided) cells within donor-derived HSC (PBS, n=8; LPS, n=9) and MPP (PBS, n=4; LPS, n=4). Data are pooled from 2-3 independent experiments. ns, not significant; *p<0.05; **p<0.01, ***p<0.001 (two-tailed t-test).

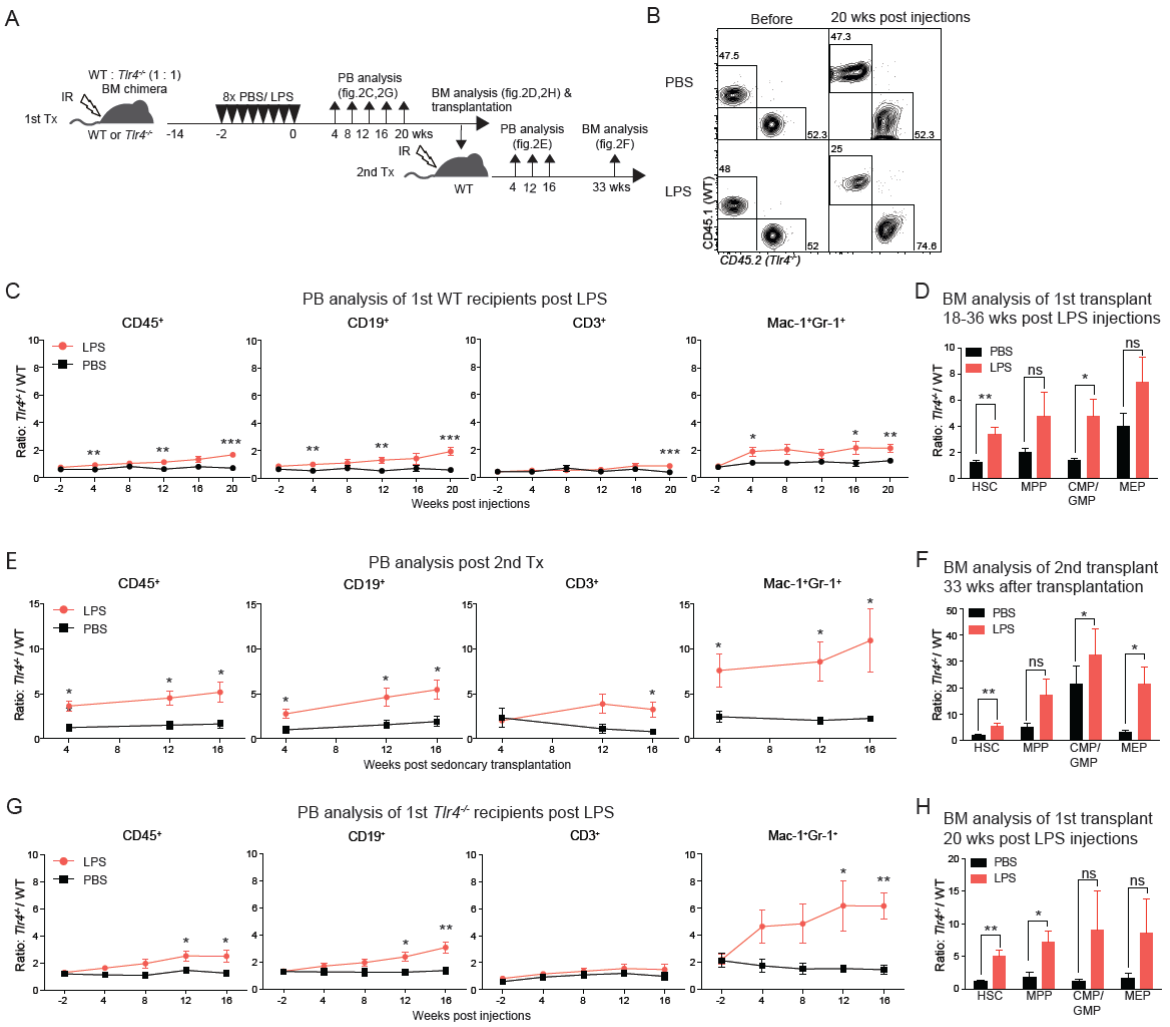


Figure 2. TLR4 activation impairs competitive self-renewing and repopulating capacity of HSC. (A) Experimental scheme of LPS challenge in mixed WT;*Tlr4*$^{-/-}$ BM chimeric animals for results depicted in (B-H): lethally irradiated WT or *Tlr4*$^{-/-}$ mice were reconstituted with WT (CD45.1^{+}) and *Tlr4*$^{-/-}$ (CD45.2^{+}) cells. Twelve weeks after reconstitution, mice were i.p. treated with PBS or LPS (35 µg) for 8 times every other day, followed by monthly PB analysis of hematopoietic chimerism, starting at 1 month after the last injection. Twenty to thirty-six weeks post transplantation, total BM cells from primary recipients were serially transplanted into lethally irradiated secondary WT recipients (CD45.1/2^{+}). Secondary recipients were assessed monthly for PB chimerism and BM was analyzed at termination of the experiment. (B) Representative flow-cytometric profiles of WT (CD45.1^{+}) and *Tlr4*$^{-/-}$ (CD45.2^{+})-derived granulocytes (Mac-1^{+}Gr-1^{+}) in WT;*Tlr4*$^{-/-}$ BM chimeric WT mice before (left) and 20 weeks after (right) PBS- (top) or LPS- (bottom) treatment. (C) Ratio of *Tlr4*$^{-/-}$- to WT-derived CD45^{+} cells, CD19^{+} B cells, CD3^{+} T cells and Mac-1^{+}Gr-1^{+} granulocytes in PB of primary WT recipients (PBS: n=6-14; LPS: n=6-13). (D) Ratio of *Tlr4*$^{-/-}$- to WT-

derived HSCs (LKSCD34$^-$), MPPs (LKSCD34$^+$), CMPs+GMPs (LKCD34$^+$) and MEPs (LKCD34$^+$) in BM of primary WT recipients 18-36 weeks post treatment (PBS: n=11; LPS: n=15). (E) Ratio of *Tlr4*$^{-/-}$- to WT-derived CD45$^+$ cells, CD19$^+$ B cells, CD3$^+$ T cells and Mac-1$^+$Gr-1$^+$ granulocytes in PB of secondary WT recipients (PBS: n=8; LPS: n=9). (F) Ratio of *Tlr4*$^{-/-}$- to WT-derived HSCs, MPPs, CMPs+GMPs and MEPs in BM of secondary WT recipients 33 weeks post treatment (PBS: n=5; LPS: n=5). (G) Ratio of *Tlr4*$^{-/-}$- to WT-derived CD45$^+$ cells, CD19$^+$ B cells, CD3$^+$ T cells and Mac-1$^+$Gr-1$^+$ granulocytes in PB of primary *Tlr4*$^{-/-}$ recipients (PBS: n=8-12; LPS: n=10-13). (H) Ratio of *Tlr4*$^{-/-}$- to WT-derived HSCs (LKSCD34$^-$), MPPs (LKSCD34$^+$), CMPs+GMPs (LKCD34$^+$) and MEPs (LKCD34$^+$) in BM of primary *Tlr4*$^{-/-}$ recipients 20 weeks post treatment (PBS: n=5; LPS: n=8). Data are pooled from 2-5 independent experiments. ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (two-tailed t-test).

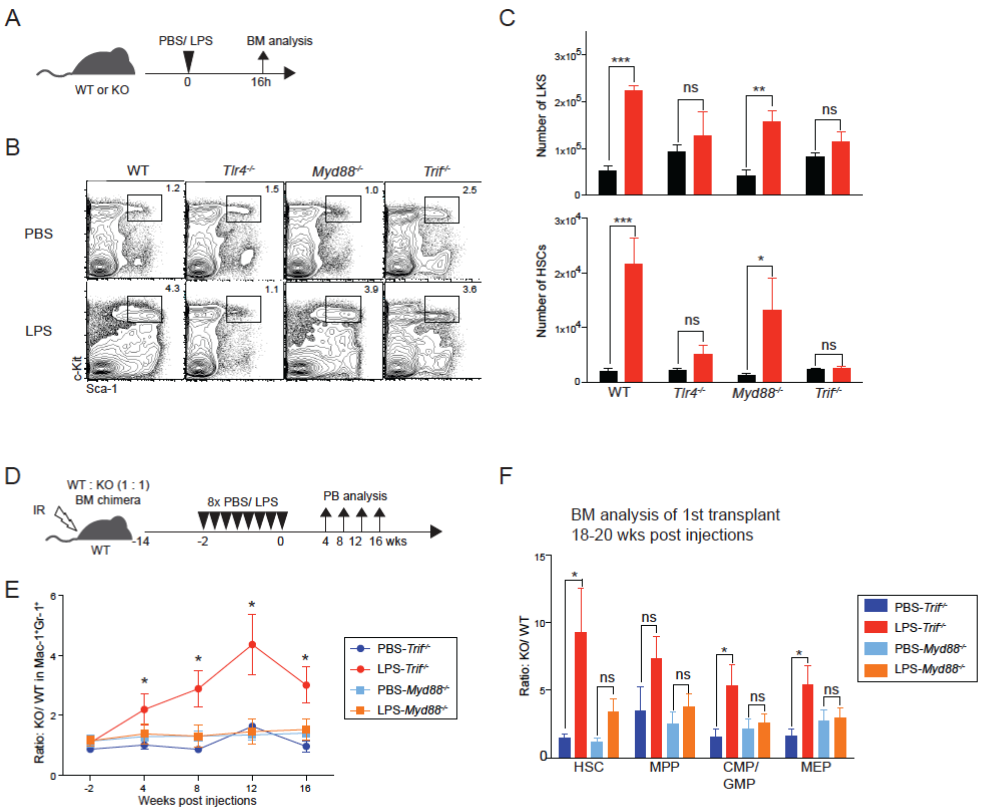


Figure 3. LPS-induced expansion and dysfunction of HSC is mediated by TLR4-TRIF signaling. (A) Experimental scheme of acute LPS challenge for results depicted in (B) and (C): WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ mice were injected with PBS or LPS (35 µg) once, and BM was analyzed 16 hours post injection for phenotypic HSC. (B) Representative flow-cytometric profiles of LKS in BM of WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ mice treated with PBS or LPS (upper and lower panel, respectively). (C) Number of LKS or phenotypic HSCs (LKSCD34^{-}CD48^{-}CD41^{-}CD150^{+}) in BM calculated from 3 independent experiments (PBS: n=3-7; LPS: n=3-6). (D) Experimental scheme of LPS challenge to BM chimeric mice for results depicted in (E) and (F): lethally irradiated WT (CD45.1/2^{+}) mice were reconstituted with WT (CD45.1^{+}) and *Myd88*$^{-/-}$ (CD45.2^{+}) or WT and *Trif*$^{-/-}$ (CD45.2^{+}) cells in a 1:1 ratio, and 3 months later treated with PBS or LPS (35 µg) for 8 times over two weeks. (E) Monthly analysis of *Trif*$^{-/-}$- *or Myd88*$^{-/-}$- to WT-derived granulocyte (Mac-1^{+}Gr-1^{+}) ratio in PB of mixed BM chimeric mice after the last PBS or LPS injection (*Trif*$^{-/-}$-PBS: n=4-11; *Trif*$^{-/-}$-LPS: n=8-13; *Myd88*$^{-/-}$-PBS: n=9-10; *Myd88*$^{-/-}$-LPS: n=10). Data are pooled from 2-5 independent experiments. ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (two-tailed t-test).

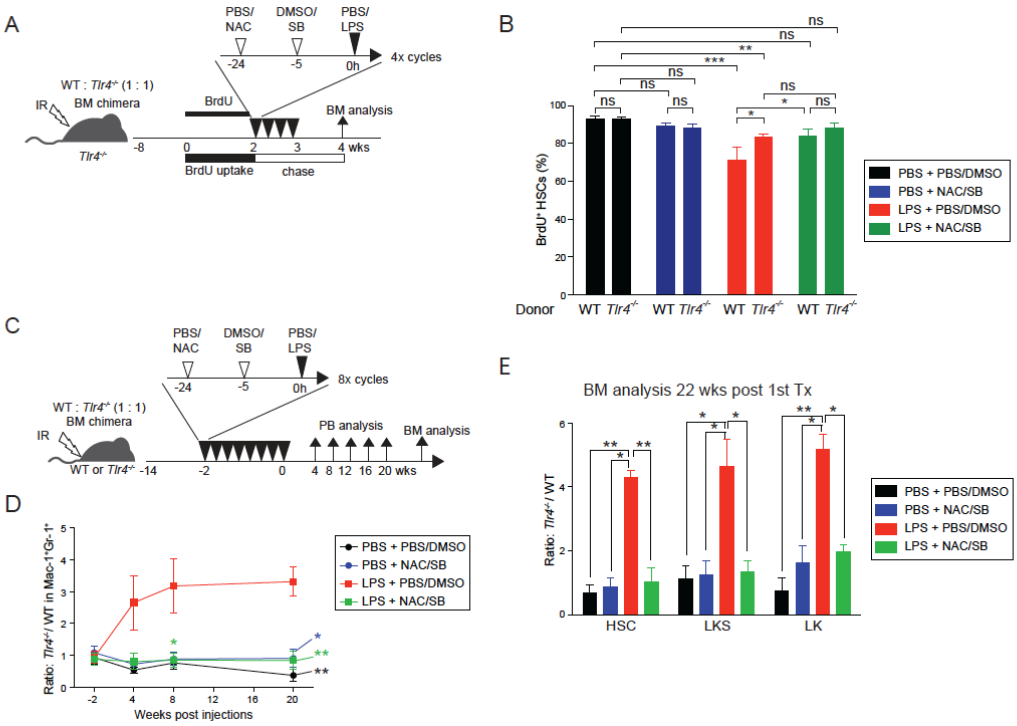


Figure 4. Pharmacological inhibition of ROS-p38 signaling prevents HSC proliferation and exhaustion upon LPS challenge. (A) Experimental scheme of BrdU label retention with or without NAC and SB for results depicted in (H): eight weeks after reconstitution of *Tlr4*$^{-/-}$ mice with WT or *Tlr4*$^{-/-}$ cells in a 1:1 ratio, mice were given BrdU containing water for 2 weeks and treated with 4 cycles of NAC (-24h) and SB (-5h) (NAC/SB) prior to each four PBS or LPS (50 µg) injections, followed by BM analysis 1 week after the last injection. (B) Percentage of BrdU^{+} HSCs (LKSCD34^{-}CD150^{+}) derived from WT or *Tlr4*$^{-/-}$ donors upon PBS or LPS injections with or without NAC and SB (PBS+DMSO: n=9; PBS+NAC/SB: n=11; LPS+DMSO: n=9; LPS+NAC/SB: n=11). (C) Experimental scheme of PBS or LPS challenge to mixed WT;*Tlr4*$^{-/-}$ BM chimeric mice, with or without NAC and SB for results depicted in (D) and (E): Twelve weeks after transplantation of WT or *Tlr4*$^{-/-}$ mice with WT and *Tlr4*$^{-/-}$ cells in a 1:1 ratio, mice were treated with 8 cycles of NAC (-24h) and SB (-5h) prior to PBS or LPS (35 µg) injections followed by monthly PB chimerism analysis and terminal BM analysis. (D) Ratio of *Tlr4*$^{-/-}$- to WT-derived granulocytes (Mac-1^{+}Gr-1^{+}) in PB of the mixed BM chimeras are shown (PBS+DMSO: n=4; PBS+NAC/SB: n=4; LPS+DMSO: n=3; LPS+NAC/SB: n=4). The ratio from PBS+DMSO, PBS+NAC/SB, and LPS+NAC/SB treated mice was statistically compared with that from LPS+DMSO treated mice. (E) Ratio of *Tlr4*$^{-/-}$- to WT-derived HSCs (LKSCD34^{-}), MPPs (LKSCD34^{+}), CMPs+GMPs (LKCD34^{+}) and MEPs (LKCD34^{+}) in BM of mixed BM chimeric mice at terminal analysis 22 weeks

post treatment (PBS+DMSO: n=4; PBS+NAC/SB: n=4; LPS+DMSO: n=3; LPS+NAC/SB: n=4). Data are pooled from 2-5 independent experiments. ns, not significant; *p<0.05; **p<0.01 (two-tailed t-test).

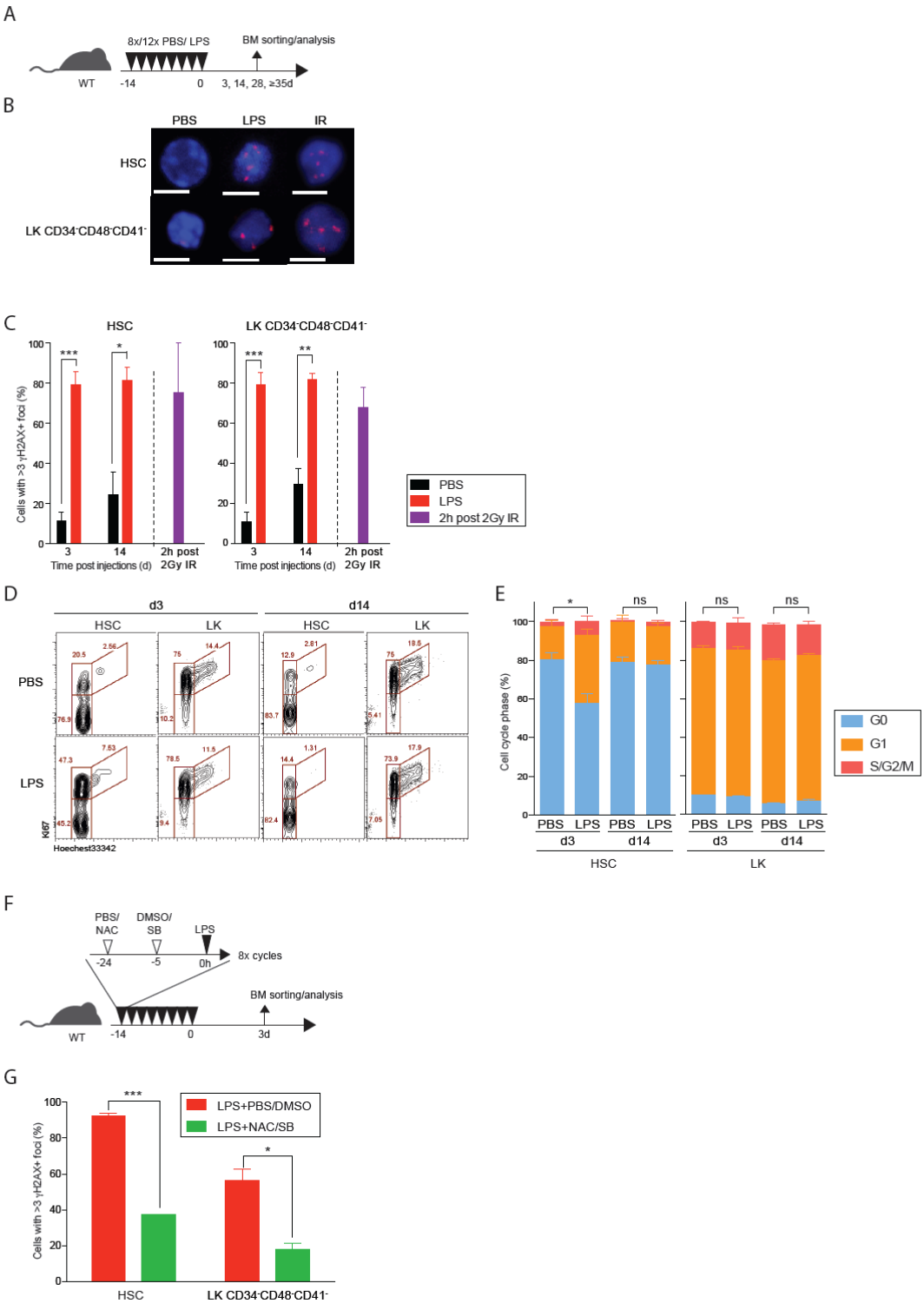
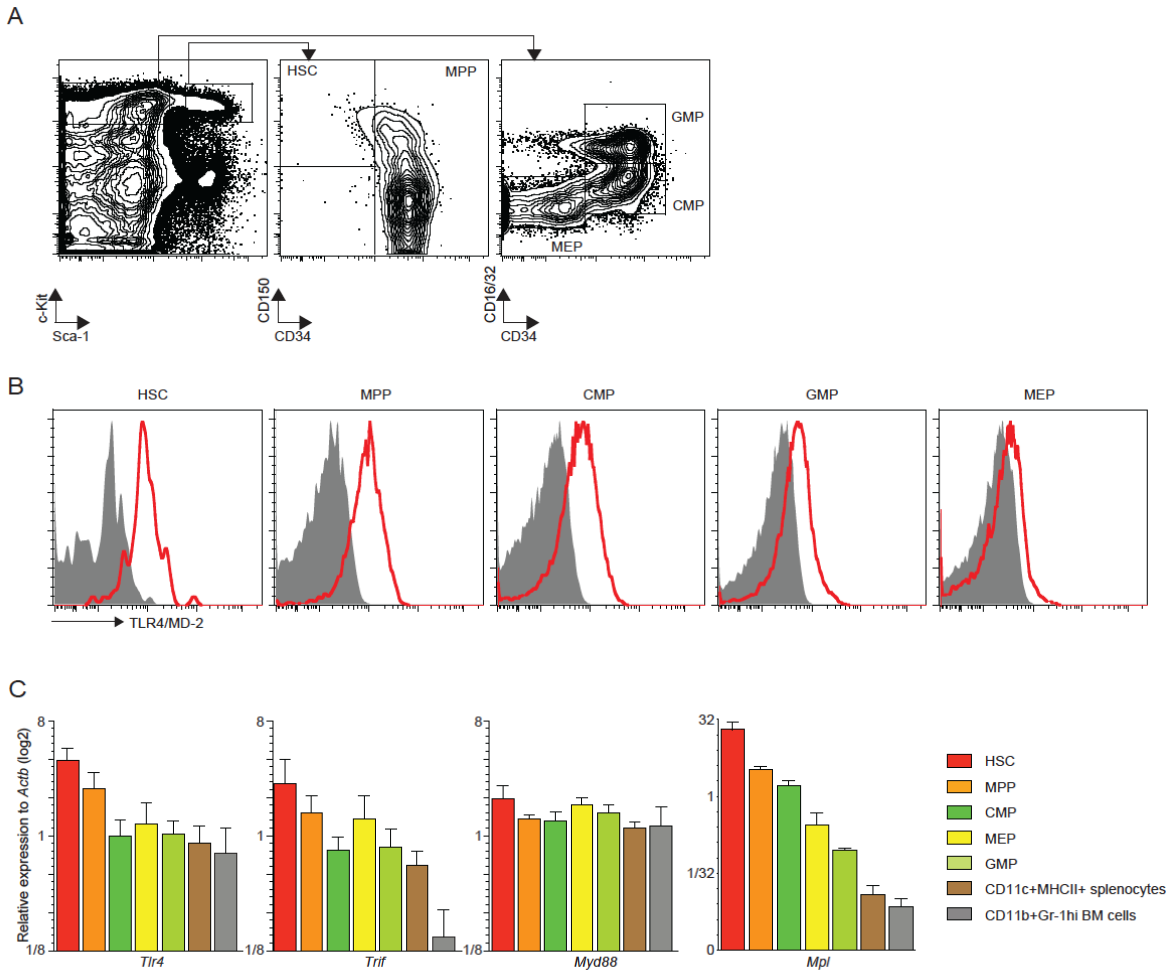


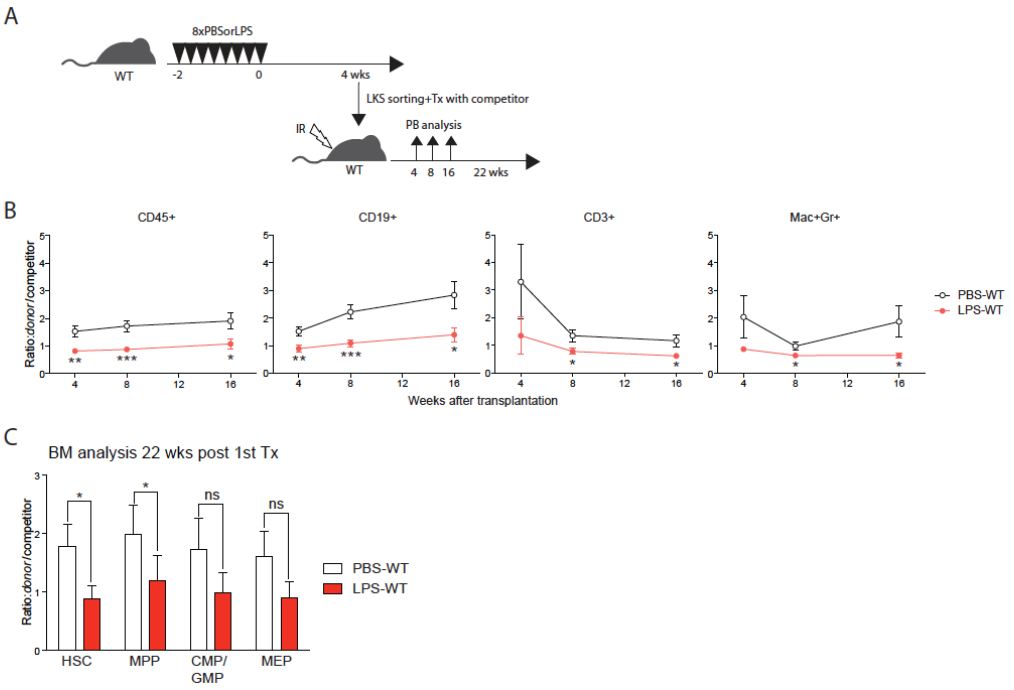
Figure 5. LPS induces DNA stress responses in HSCs via ROS-p38 activation. (A) Experimental scheme of sustained LPS challenge for results depicted in (B-E): WT mice were treated with PBS or LPS (50 µg) 8 or 12 times every other day, and BM were processed for cell cycle analysis or sorting at different time points (3, 14, ≥35d) after the last injection. (B) Representative confocal microscopic images of co-localization of γH2AX$^+$ (red) foci in nuclei (blue) of HSCs (LKSCD34$^-$CD48$^-$CD41$^-$CD150$^+$) and LK CD34$^-$CD48$^-$CD41$^-$ cells at day 3 after PBS or LPS treatment or at 2

hours after 2Gy irradiation. White bars indicate 5 µm. (C) Time course kinetics of ▪H2AX^{+} foci formation in HSCs and LK CD34^{-}CD48^{-}CD41^{-} cells from PBS- (blue) or LPS- (red) treated mice, in comparison with those from animals 2 hours after 2Gy irradiation (green). The percentage of cells with >3 γH2AX^{+} foci is shown. The number of PBS or LPS injections is indicated above each bar. (D) Representative flow-cytometric cell cycle analysis of HSCs (LKS CD34^{-}CD150^{+}) and LK cells at 3 and 14 days after the last injection. (E) Percentage of each cell cycle phase (G0, G1 and S/G2/M) of HSCs and LK cells (PBS: n=4-5; LPS: n=4-5 at each time point). (F) Experimental scheme of DNA damage response upon LPS application in the presence or absence of NAC and SB for results depicted in (G): WT mice were treated with 8 cycles of NAC (-24h) and SB (-5h) in prior to LPS injections. (G) The percentage of cells with >3 γH2AX^{+} foci in HSCs and LK CD34^{-}CD48^{-}CD41^{-} cells 3 days after the last LPS injection with or without NSC/SB. Data are pooled from 2-4 independent experiments. n.d., not determined; ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (two-tailed t-test).

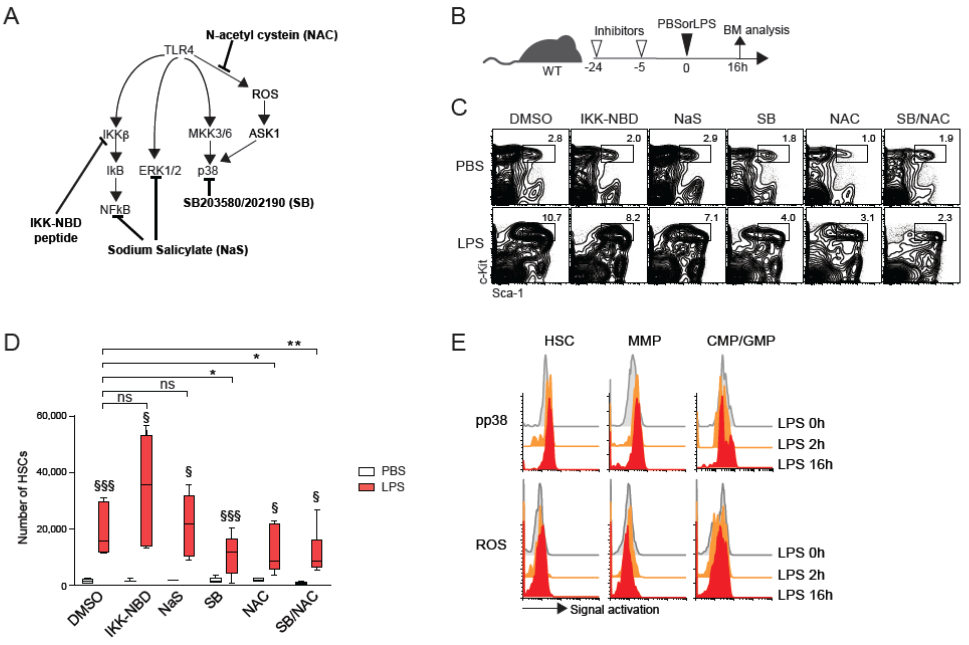
Supplemental Information



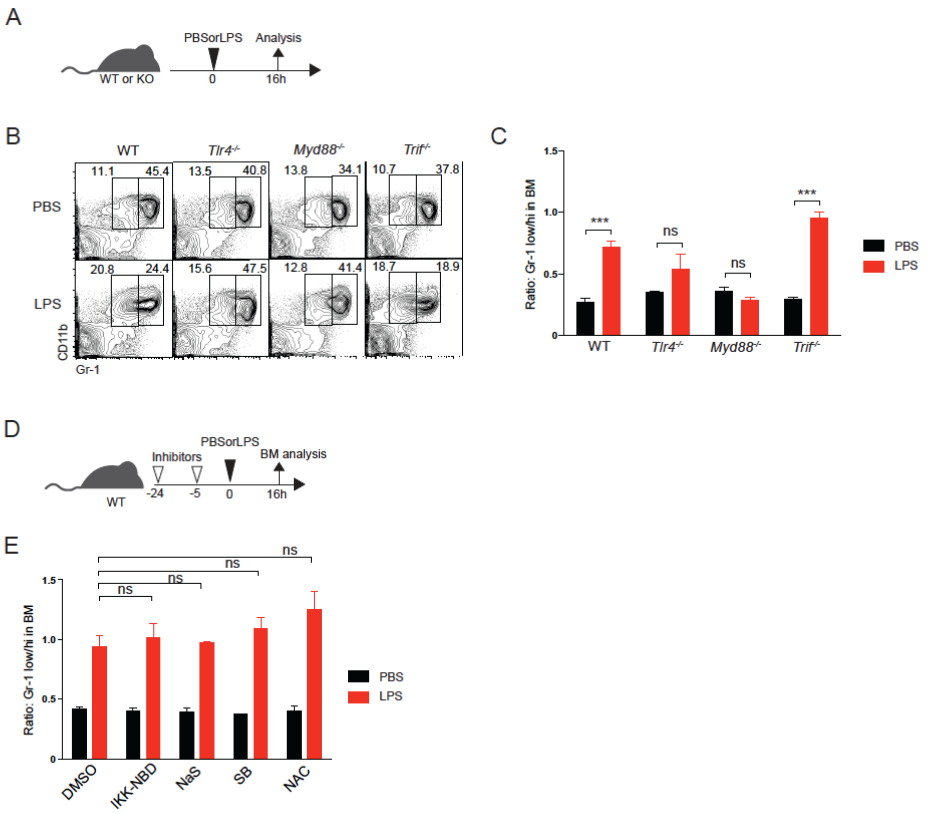
Supplementary figure 1. HSC and progenitors express TLR4/MD-2 complex. (A) Representative flow-cytometric profiles of HSCs and progenitors in BM of WT mice. Lin$^-$ BM cells were pre-gated and developed by c-Kit and Sca-1, and subsequently CD150 and CD34, or CD16/32 and CD34, to define the following populations: HSCs (LKSCD34$^-$CD150$^+$), MPPs (LKSCD34$^+$), CMPs (LKCD16/32^{low}CD34$^+$), GMPs (LKCD16/32^{hi}CD34$^+$) and MEPs (LKCD16/32^{low}CD34$^-$). (B) Representative histogram of TLR4/MD-2 expression in HSCs, MPPs, CMPs, GMPs and MEPs (red: anti-TLR4/MD2 antibody; gray: isotype-matched antibody). (C) Quantitative PCR analysis on mRNA expression of the indicated genes in FACS-sorted BM HSCs (LKS Flt3$^-$CD34$^-$CD48$^-$CD150$^+$), MPPs (LKS Flt3$^+$CD34$^+$), CMPs, MEPs (LKCD16/32^{low}CD34$^-$), GMPs (LKCD16/32^{hi}CD34$^+$), dendritic cells (CD11c$^+$MHCII$^+$) from spleen and granulocytes (Mac1$^+$Gr1$^+$) from BM. Relative expression of the indicated genes to the expression of *Actb* is shown in respective cell populations (mean ± SEM). Data are pooled from 2-4 independent experiments.



Supplementary figure 2. Systemic LPS application limits competitive repopulating ability of HSCs, irrespective of LPS-induced HSC mobilization. (A) Experimental scheme of LPS treatment and transplantation for results depicted in (B) and (C): WT or *Tlr4*$^{-/-}$ mice were i.p. injected with PBS or LPS (35µg) 8 times every other day. Four weeks after the last injection, 3,000 LKS (CD45.2^{+}) were isolated, and competitively transplanted with 3,000 non-stimulated competitor LKS (CD45.1^{+}) into lethally irradiated WT mice (CD45.1/2^{+}) followed by monthly PB chimerism analysis (B) and terminal BM analysis (C). (B) Percentage of donor derived CD45^{+} cells, CD19^{+} B cells, CD3^{+} T cells and Mac-1^{+}Gr-1^{+} granulocytes in PB of recipients (PBS-WT: n=12; LPS-WT: n=14; LPS-WT: n=9). (C) Percentage of donor derived HSCs (LKSCD34^{-}), MPPs (LKSCD34^{+}), CMPs/GMPs (LKCD34^{+}) and MEPs (LKCD34^{+}) in BM of recipients at terminal analysis 22 weeks after reconstitution (PBS-WT: n=6; LPS-WT: n=7). Data are pooled from two independent experiments. ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (two-tailed t-test).



Supplementary figure 3. Pharmacological inhibition of ROS-p38 signaling blocks expansion of phenotypic HSCs upon LPS challenge. (A) Schematic TLR4 signaling cascades, leading to MAPKs and NFκB activation that are blocked by the respective indicated inhibitors. (B) Experimental scheme of single LPS challenge with or without inhibitors for results depicted in (C-E): WT mice were pretreated with vehicle or indicated inhibitors, 24 and 5 hours prior to single PBS or LPS (35 µg) injection. BM was analyzed 16 hours post PBS or LPS injections. (C) Representative flow-cytometric profiles of c-Kit and Sca-1 expression in Lin^- BM cells are shown. (D) Number of HSCs ($LKSCD34^-CD48^-CD41^-CD150^+$) were calculated (PBS, n=3-6; LPS, n=5-9). Data from LPS applications with inhibitor were statistically compared to those from LPS with DMSO: *p<0.05; **p<0.01 (two-tailed t-test); Data from LPS application with or without inhibitor were statistically compared with those from their corresponding PBS control: *p<0.05; **p<0.001 (two-tailed t-test). (E) Representative histogram of p38 and ROS activation in HSCs ($LKSCD34^-$), MPPs ($LKSCD34^+$) and CMPs+GMPs ($LKCD34^+$) upon LPS challenge: cells were isolated from BM at 0, 2 and 16 hours post single PBS or LPS (35 µg) injection, and tested for p38 and ROS activation.



Supplementary figure 4. Emergency granulopoiesis is dependent on MYD88 but not TRIF-ROS-p38 mediated pathways. (A) Experimental scheme of the acute LPS challenge of mice deficient for TLR4 signal-related molecules for results depicted in (B) and (C): WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ mice were injected with PBS or LPS (35 µg), and analyzed for BM granulopoiesis 16 hours post injection. (B) Representative flow-cytometric profiles of CD11b and Gr-1 expression in BM from WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ mice treated with PBS (upper) or LPS (lower) panel. (C) Ratio of CD11b^{+}Gr-1^{low} to CD11b^{+}Gr-1^{hi} cells in BM (PBS: n=3-5; LPS: n=3-5) (D) Experimental scheme of acute LPS challenge with or without inhibitors for results depicted in (E): WT mice were pretreated with vehicle or the respective indicated inhibitors, 24 and 5 hours prior to single PBS or LPS (35 µg) injection. (E) Ratio of CD11b^{+}Gr-1^{low} to CD11b^{+}Gr-1^{hi} cells in BM (PBS: n=3-5; LPS: n=3-5 for each inhibitor) Data are pooled from 3 independent experiments. ns, not significant; ***p<0.001 (two-tailed t-test).

Supplemental Extended Experimental Procedures

Mice

CD45.1/2^{+} mice were generated by intercrossing C57BL/6 (CD45.2^{+}) with B6.SJL (CD45.1^{+}). *Tlr4*$^{-/-}$ mice were backcrossed on C57BL/6 (CD45.2^{+}) or B6.SJL (CD45.1^{+}) for more than six generations, and CD45.1/2^{+} *Tlr4*$^{-/-}$ mice were generated by interbreeding of both mice. All mice were maintained at the Institute for Research in Biomedicine and University Hospital Zurich animal facilities according to the guidelines of the Swiss Federal Veterinary Office. Experiments were approved by the Dipartimento della Sanità e Socialità, Ticino, Switzerland, and by the Veterinäramt of Kanton Zurich, Zurich, Switzerland.

Generation of BM chimeric mice, LPS challenge and serial transplantation

Six to twelve week old WT or *Tlr4*$^{-/-}$ mice (CD45.1/2^{+}) were lethally irradiated with 9.5Gy (split dose within a 2-4 h interval) and i.v. transplanted with equal numbers (1:1 ratio) of 1x10^{6} total BM or 3,000 Lin^{-}c-Kit^{+}Sca-1^{+} cells from WT and *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, or *Myd88*$^{-/-}$ animals. Two to three months after reconstitution, the BM chimeric mice were i.p. injected with 35-50 µg LPS corresponding to 35-50 EU (*Escherichia coli 0111:B4*, LPS-EB Ultrapure, Invivogen) according to the indicated schemes. Peripheral blood was harvested from the BM chimeric mice and the relative donor chimerism was assessed in mature hematopoietic cells. At terminal work-up, BM from chimeric mice was harvested and analyzed.

FACS analysis and sorting

All antibodies used in this study were purchased from Life technologies (eBiosciences) or Biolegend unless specified otherwise. For analyzing donor chimerism in mature hematopoietic cell, cells from BM, spleen, or peripheral blood (PB) were stained with antibodies against CD45.1 (A20), CD45.2 (104), CD19 (1D3), CD3▪ (2C11), Gr-1 (RB6-8C5) and CD11b (M1/70). For early hematopoietic cell analysis, cells from BM or spleen were incubated with antibodies against lineage (Lin) markers (NK1.1 (PK136), CD11b, Ter119 (Ter119), Gr-1 (RB6-8C5), CD4 (RM4-5), CD8α(53-6.7), CD3ε (2C11), B220 (RA3-6B2), IL-7Rα (A7R34), c-Kit (2B8), Sca-1 (D7), CD34 (RAM34), Flt3 (A2F10), CD150 (TC15-12F12.2), CD48 (HM48-1),

CD16/32 (2.4G2). For LKS or HSC (LKSCD34^{-}CD48^{-}CD150^{+}) sorting, total BM cells were stained with biotinylated antibodies against lineage markers, and lineage positive cells were depleted with streptavidin conjugated magnetic beads (Militenyi Biotec GmbH). The lineage depleted cells were stained with antibodies against c-Kit and Sca-1, and LKS or HSC were sorted on a FACS Aria III (BD Biosciences). For TLR4 expression, total BM cells were incubated with biotinylated an antibody against TLR4/MD-2 complex (MTS510, Life technologies) or an isotype-matched control antibody, together with early hematopoietic cell markers (Lin, c-Kit, Sca-1, CD34, CD150, CD16/32) for more than 90 min, and binding was detected by streptavidin conjugated brilliant violet 421. The samples were analyzed on a FACS Canto II.

Quantitative RT-PCR

HSC and HPC from BM were enriched for Lin^{-} cells with biotin-conjugated lineage markers as described above. Cells were then stained with the antibodies against CD16/32 (2.4G2), c-Kit (2B8), Sca-1 (D7), Flt3 (A2F10), CD34 (RAM34), CD150 (TC15-12F12.2). For mature cell populations, BM and spleen cells were stained with Mac1 (M1/70) and Gr-1, and MHCII (M5/114.15.2), CD11c (N418), respectively, and were directly sorted into lysis buffer containing β-mercaptoethanol. The cell lysate was subjected to RNA isolation (Qiagen), cDNA synthesis (Applied Biosystems) and qPCR with SYBER green reagent (Applied Biosystems) and the following gene specific primers on a 7500 Fast Real Time PCR System (Applied Biosystems): *Tlr4* (5'-CCA ATG CAT GGA TCA GAA ACT C-3' ; 5'-ATT TCA CAC CTG GAT AAA TCC AGC-3'), *Trif* (5'-TAC AGC CAG GTC TGT GCT-3' ; 5'-AGA ATG AAG CCT GGA GCC-3'), *Myd88* (5'-TCG ATG CCT TTA TCT GCT ACT G-3' ; 5'-TCT GTT GGA CAC CTG GAG AC-3'), *Mpl* (5'-GAA GCT GTC TCG TCT CAG G-3' ; 5'- TCC AAT TGT CAC TGC ATC TCC-3') and *Actb* (5'-AGA TGA CCC AGA TCA TGT TTG AG-3' ; 5'- GTA CGA CCA GAG GCA TAC AG-3'). Relative mRNA expression of each gene was normalized against relative expression of beta-actin.

***In vivo* inhibitor treatment**

For inhibitor treatment, BM chimeric mice were i.p. injected with 100 mg/kg NAC (Calbiochem, CA, USA) for ROS, 15 mg/kg SB203580 or SB 202190 (Calbiochem) for p38, 2 mg/kg of IKK-NBD peptide (Enzo Life Sciences) for NFκB, 200 mg/kg

Sodium Salicylate (Calbiochem) for NFκB or the respective vehicle, 24 and 5 hours prior to PBS or LPS administration.

BrdU labeling and retention

WT or mixed BM chimeric mice were i.p. injected with 9 mg/kg BrdU and subsequently put on drinking water containing 800 µg/ml BrdU and 5% sucrose for two weeks with refreshing the water every 2-3 days. Then drinking water was switched to normal water for another two weeks until analysis. While given normal drinking water, animals were treated with PBS or LPS, together with or without inhibitors as described above. One week after final PBS or LPS injections, BM cells were harvested and subjected to cell surface staining with antibodies against lineage markers above, and to intracellular anti-BrdU staining with an APC conjugated antibody according to the manufacturer's instructions (BD bioscience). BrdU positivity was determined by measuring baseline intensity using non-BrdU treated BM, stained with the anti-BrdU antibody.

Detection of ROS and p38 activation

HSCs (LKS $CD34^-CD150^+$), MPPs (LKS $CD34^+$), and CMPs/GMPs (LK $CD34^+$) were sorted from BM of WT mice that were treated with PBS or 50 µg LPS 16 hours before, and incubated with 10 µM DCF-DA (Life technologies) for 30 min at 37°C, followed by fixation with 2% PFA for 10 min. After permeabilization with 0.1% Triton X-100 (Roche)/PBS for 20 min and blocking with 0.1% Triton X-100/PBS containing 10% goat serum (DAKO) for an hour, the cells were stained with anti-phospho p38 (Cell signaling) and detected by goat anti-rabbit AlexaFluor 488 (Life Technologies). Cells were analyzed on a FACS Canto II, and positivity for each marker was determined based on the intensity of PBS-treated cells that were stained with both markers.

CFSE labeling and chasing

WT HSCs (3,000-7,835 LKS $CD34^-CD48^-CD41^-CD150^+$ cells) or MPPs (113,250 LKS $CD34^+$ cells) were isolated from B6/J mice ($CD45.1^+$), labeled with CFSE as previously described (Takizawa JEM 2011) and i.v. transferred into *Tlr4*$^{-/-}$ mice ($CD45.1^+$) without irradiation. Two or three days after mice were treated with PBS or 100 µg LPS for 2 or 3 times and were analyzed on day 6 for HSCs and MPPs.

Immunocytochemistry

HSC (LKS $CD34^{-}CD48^{-}CD41^{-}CD150^{+}$) and LK $CD34^{-}CD48^{-}CD41^{-}$ cells were sorted from BM of WT mice at 3 or 14 days after i.p. injection of PBS or 50 µg LPS for 8 or 12 times, and plated on a glass slide, coated with 0.01% poly-L-lysine (Sigma Aldrich), followed by 60 min incubation at R.T (Room Temperature) to let cells settle down on the slide. Cells were then fixed with 4% PFA for 10 min and permeabilized with 0.5% Triton X-100 (Roche)/PBS for 20 min and blocked with 0.5% Triton X-100/PBS containing 10% goat serum (DAKO) for one hour or overnight. Anti-phospho-histone H2AX (JBW301, Millipore) staining was done overnight at 4°C, and subsequently binding was visualized with a goat anti-mouse AlexaFluor 647 (Life Technologies) for the respective primary antibodies. Nuclei were counterstained with DAPI (Life Technologies) and images were taken on a Leica SP5 with a 63x objective lens (Leica). The number of phosphor-H2AX positive foci was counted manually on image software, Imaris (Bitplane, USA).

RESEARCH ARTICLE 2

(in preparation)

Engineered human bone organs maintain human hematopoiesis *in vivo*

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Key points:

- BM-derived human MSCs can form a hematopoietic niche through endochondral ossification
- Functional human hematopoietic stem and progenitor cells are maintained within the human bone organ

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Abstract (200 words allowed)

Hematopoietic stem cells (HSCs) are maintained in a so-called bone marrow (BM) niche, which is a specialized environment that provides factors for their maintenance. While the cellular and molecular components of the mouse BM niche have been studied extensively, little is known about the human BM niche components. We have previously shown with a developmental tissue engineering approach that human adult BM mesenchymal stromal cells (MSCs) can *ex vivo* generate a human cartilage template and to *in vivo* develop human bone organs, so called “ossicles”. However, it has not been tested whether the human bone organs derived from MSCs can function as niche for human HSC maintenance. Upon *in vivo* implantation of the human ossicles into immunodeficient mice and engraftment of human CD34+ cells, we show an efficient development of human hematopoiesis with phenotypic HSCs in the human ossicles. Functional assays show a better maintenance of human hematopoietic stem and progenitors within the human ossicles compared to mouse bone marrow, proving that the human ossicle can serve as a functional human BM niche. The engineering of a heterotrophic human BM niche that is transplantable and re-engineerable can serve as a platform that allows the study of human hematopoiesis in an optimized environment *in vivo*.

Introduction

Lifelong self-renewal and multilineage repopulation capacity of hematopoietic stem cells (HSCs) is maintained in a specialized microenvironment in the bone marrow (BM), the so-called “niche” that provides HSCs with vital factors for their maintenance. Besides the hematopoietic cells, the BM microenvironment is composed of a complex network of cells, such as adipocytes[35], osteoblast/osteoclast cells[36-39], megakaryocytes[33,34,133] and endothelial cells[40,41,45], macrophages[31,32,45] and Schwann cells[42], as well as specialized stromal cells as e.g. Nestin-positive mesenchymal stromal cells (MSCs)[43], CXCL12 abundant reticular cells (CARs)[44,45,47], and leptin receptor-positive cells (LepR+ cells)[45]. However, little is known about the cellular and molecular components of the human BM niche. In recent years the field has taken great leaps in engineering the BM microenvironment by genetic modification of animals as mice. This approach has some limitations e.g., it is time-consuming, results usually in one modification at a time and is restricted to mice or surrogate organisms that do not necessarily reflect the human situation. As MSCs are able to differentiate into chondrocytes through endochondral ossification, they are used as a source to form bone marrow microenvironment *in vivo*[95]. Several studies have suggested that upon subcutaneous implantation of mouse or human MSCs into immune-deficient mice they are able to form a supportive bone marrow cavity through a vascularized cartilage intermediate which was replaced by hematopoietic tissue and bone and that can attract and support murine hematopoiesis[93,97-99]. Nevertheless there is a lack of *in vivo* models that can recapitulate the complexity of a human BM environment to support normal and malignant hematopoiesis. Thus, the current xenotransplant mouse models are not able to reproduce the physiological interactions between HSCs and a human BM microenvironment as human HSCs are engrafted in a mouse BM microenvironment. A recent study has shown that only human BM-derived MSCs are able to form a bone marrow cavity through a vascularized cartilage intermediate[100], which can maintain murine long-term HSCs as well as functional human HSCs defined as CD34+CD38-CD90+CD45RA+ after cord blood transplantation[101]. Most of the recent developmental tissue engineering protocols [55] do not or only in a small amount show the mineralization of the newly formed tissue, which does not mimic a fully regenerated bone organ. Recently we reported a tissue engineering protocol where human adult BM-derived mesenchymal

stromal cells (MSCs) are *ex vivo* differentiated into hypertrophic cartilage, and, upon *in vivo* implantation into immunodeficient mice, develop to a fully mature bone organ, so called „ossicle“, via endochondral ossification[99]. We hypothesize that these ossicles provide a sufficient engraftment and maintanance of human hematopoiesis in a xenocraft mouse model.

Here we show that indeed human ossicles can serve as a niche that can better maintain functional human Hematopoietic stem cells (HSC) compared to the mouse BM of immune-compromised hTPOhSIRP mice.

Material and Methods

Mice

The human TPO knock-in replacement was performed by using Velocigene Technology , as reported previously[134]. Human SIRPα expression was achieved by transgenesis using a BAC in the same genetic background, as previously described[135]. These two mouse strains were intercrossed to generate Balb/c Rag2 $IL2^{-/-}$ cγ-/- $hTPO^{KI}$ $hSIRP\alpha^{Tg}$ mice (STRG). All mice were maintained at the University Hospital Zurich animal facility according to the guidelines of the Swiss Federal Veterinary Office, and all the experiments were approved by the Veterinäramt of Kanton Zurich, Zurich, Switzerland.

MSC isolation, *in vitro* culture, and *in vivo* implantation.

Human mesenchymal stem cells (MSCs) were isolated from human bone marrow aspirates and processed as previously reported[99]. Shortly, MSCs were expanded for maximum four passages and seeded onto type I collagen meshes (8-mm-diameter, 2-mm-thick disks; Ultrafoam, Davol) at a density of 70×10^6cells/cm^3 and cultured in a serum-free chondrogenic medium for 3 weeks, followed by 2 wk in a serum-free hypertrophic medium, supplemented with 50 nM thyroxine, 7.0×10^{-3}M β-glycerophosphate, 10^{-8}M dexamethasone, and 2.5×10^{-4}M ascorbic acid and IL-1β (50 pg/mL). The resultant human cartilage templates were implanted in s.c. pouches on the back of 6-10 week old Balb/c Rag2 $IL2^{-/-}$ cγ-/- $hTPO^{KI}$ $hSIRP\alpha^{Tg}$ mice (four samples per mouse).

Human progenitor cell isolation and injection

Human CD34+ cells were purified from cord blood by density gradient centrifugation followed by positive immunomagnetic selection with anti-human CD34 microbeads (Miltenyi Biotec). Cells were frozen in FBS containing 10% DMSO and kept in liquid nitrogen. All human studies were approved by the Cantonal ethics committee of Zurich, Switzerland. Four weeks after ossicle implantation, hTPOhSIRP mice were sublethally irradiated (400 cGy) and 6-8x10^5 CD34+ cells pooled from several donors were injected intravenously, followed by analysis at 8 weeks after transplantation.

Flow cytometry

Mice were euthanized, and vascularized ossicle as well as mouse Femur and Tibia were removed. Explanted human ossicle and mouse bone were crushed using a mortal and pestle, digested at 37°C for 45 min in DMEM (Invitrogen), 10% FCS (Invitrogen), 10mM HEPES (Invitrogen), 0.4% collagenase II (Worthington) and 0.02% DNase I (Worthington) and washed with PBS containing 2% human Serum and then filtered on a 70 µm cell strainer. The resultant cells were incubated with human and mouse FcR blocking antibodies (Miltenyi Biotec) and stained with TRI-COLOR conjugated human lineage antibodies (CD2, CD3, CD4, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, CD235a; Invitrogen), human CD45-eFluor 450 (clone HI30, eBioscience), human CD90-PE (clone 5E10, Beckton Dickinson (BD)), human CD38-FITC (clone HIT2, BD), human CD34-PECy7 (clone 8G12, BD), human CD45RA-APC eFluor 780 (clone HI100, eBioscience) and mouse CD45.2-APC (clone 104, Biolegend). Multi-lineage engraftment in peripheral blood (PB) and cells from human ossicle and mouse bone, was detected by human CD3-PECy7 (clone UCHT1, eBioscience), human CD19-APCCy7 (clone HIB19, BD) and human CD33-APC (clone P67.6, BD), human CD45-eFluor 450 (clone HI30, eBioscience) and mouse CD45.2-FITC (clone 104, eBioscience). Propidium Iodide (Invitrogen) was added to the samples to exclude dead cells. All samples were analyzed on a FACS Aria III (BD Biosciences) or LSR Fortessa (BD Biosciences).

CFU and Serial transplantation

For the colony-forming assay, 1000 hCD45+hlin-hCD34+ cells were sorted from ossicle and femur bone marrow, respectively, and plated onto methylcellulose medium (Stem Cell Technologies) containing 100 ng/ml hIL-3, 50 ng/ml hIL-6, 50 ng/ml hIL-11, 50 ng/ml hSCF, 250 ng/ml hTPO, 20 U/ml hEPO, 250 ng/ml hGM-CSF and 50 ng/ml hFlt3L. Cultures were maintained at 37°C in 5% CO_2 and scored after 12-14 days.

For serial transplantation, 4 x10^5 CD34+ cells from ossicle and femur bone marrow were serially transplanted into sublethally irradiated hTPOhSIRP mice, followed by monthly PB and terminal BM analysis for human engraftment.

Cell cycle analysis assay

Single cell suspensions from human ossicle and mouse bone were first stained with TRI-COLOR conjugated lineage antibodies, human CD45-BV711 (clone HI30,

Biolegend), mouse CD45-BV786 (clone 30-F11, Biolegend), human CD90-FITC (clone 5E10, BD), human CD38-APC (clone HIT2, BD), human CD34-PECy7 (clone 8G12, BD), human CD45RA-APC eFluor 780 (clone HI100, eBioscience) to detect human hematopoiesis. After washing with PBS containing 2% human Serum cells were fixed with Fixation/Permeabilization buffer (eBioscience) for 30min at 4°C. Cells were then stained with PE-anti Ki67(dilution 1:5, BD) for 45 min at room temperature in Permeabilization buffer (eBioscience). After washing, cells were stained with DAPI (1mg/ml, Invitrogen) for 30 min at 4 °C and immediately analysed on a LSR Fortessa (BD Biosciences).

Histological Staining

Samples were embedded in paraffin and sections of 5 µm thickness prepared using a microtome. Safranin-O, Alizarin red, hematoxylin/eosin and Masson tri-chrome staining were performed as published previously[99].

Microtomography

Samples were collected and fixed overnight in 1.5% formaldehyde at 4°C. Microtomography of the explants was performed using a tungsten x-ray source at 70 kV and 260 µA with an aluminum filter of 0.5 mm (Nanotome, GE, USA). Transmission images were acquired for 360° with an incremental step size of 0.25°. Volumes were reconstructed using a modified Feldkamp algorithm (software supplied by manufacturer) at a voxel size of 2.5-3 µm. Thresholding, segmentation and 3D measurements were performed using the VGStudio Max software. After microtomography, samples were decalcified in 15% EDTA solution (Sigma Aldrich) before histology.

Statistical analysis

All data are shown as the mean, unless indicated otherwise. All statistical comparisons were evaluated with Students t-Test; *$p<0.05$; **$p<0.01$; ***$p<0.001$ (two-tailed t-test).

Results and Discussion

Human hematopoiesis is maintained in engineered human ossicles

Human ossicles were generated as described previously[99] and implanted subcutaneously in the back of immune-compromised mice (hTPhSIRP). Four weeks after implantation, mice were irradiated and transplanted with human cord-blood (CB)-derived CD34+ cells. After 2 months, the engraftment of human HSPCs was analyzed in the human ossicle and mouse bone of the implanted mice by flow cytometry (Figure 1A).

Upon *in vivo* implantation, the hypertrophic cartilage underwent extensive remodeling. Hypertrophic cartilage areas are replaced by BM and bone at increasing densities of mineralization and the central construct core is filled with matrix that remodels to trabecular-like bone structures (Supplemental Figure 1). Human ossicles show efficient vascularization as indicated by reddish color (Figure 1B), suggesting homing of cells through peripheral blood circulation. Histological and micro-CT analysis confirms that the human ossicle has morphological and structural similarities to naive bone tissue.

Two months after reconstitution with human CB-derived CD34+ cells, we identified mature T cells, myeloid cells, B cells as well as immature B cells, Pro and Pre B cells in the human ossicles (Figure 1 C). As already shown in previous studies[101] the majority of B cells were immature B cells. Besides human mature cell engraftment we also detected human HSCs (CD34+CD38-CD90+CD45RA-), multipotent progenitor cells (MPP, CD34+CD38-CD90-CD45RA-) and multilymphoid progenitors (MLP, CD34+CD38-CD90+CD45RA+) within the HSPC compartment (CD34+CD38-), as well as common myeloid progenitors, megakaryocyte/erythrocyte progenitors (CMP/MEP, CD34+CD38+CD45RA-) and granulocyte/macrophage progenitors (GMP, CD34+CD38+CD45RA+) within the Myeloid progenitors compartment (MyelP, CD34+CD38+) in all human ossicles analyzed (Figure 1D and E). Interestingly we found a significant lower engraftment of myeloid progenitors including GMPs within the human ossicle niche. Although we did not observe a significant difference in the engraftment of human hematopoietic stem and progenitor cells in the human ossicles compared to the mouse bone the fold change of the frequency within one mouse showed a higher frequency of HSPCs, MPPs, HSCs, MLPs and a significant lower frequency of Myeloid Progenitors and GMPs within the human ossicle (Figure 1F).

Human ossicle niche maintains human HSPC quiescence

In mouse, mouse HSCs become quiescent through the interaction with the surrounding niche. In contrast, human HSCs in a xenograft mouse model are highly proliferative[136], suggesting that the mouse niche does not support human HSC quiescence. To investigate whether the human ossicle can provide a human HSC niche that's drives them towards quiescence, we performed a cell cycle analysis of HSPCs within the human ossicle and the mouse bone. We observed that overall less than 5 % of Hematopoietic cells are quiescent compared to CB-MNC with up to 90% quiescent cells (Figure 2A). Nevertheless, there is a tendency that HSPCs, MyelP, HSCs and MLPs are more quiescent in the human ossicle compared to the mouse bone (Figure 2B). Interestingly more than 50% of HSCs are in S-G2-M phase whereas only 10 % of HSPCs, MyelP, MPPs and MLPs are in S-G2-M phase (Supplementary Figure 3). These results suggest that HSPC as well as MyelP become more quiescent through the interaction with the human ossicle niche.

Functional, self-renewing human HSPCs are maintained in human ossicle

Finally, to investigate if HSPCs that are maintained in the human ossicles are also functional, we first performed a Myeloid-colony forming unit (CFU) assay. Out of 1000 plated hCD45+hlin-hCD34+ from mouse bone and human ossicle, the cells from the human ossicle gave rise to significant more myeloid colonies compared to the once from the mouse bone. This suggests that there is a higher frequency of functional hematopoietic stem and progenitor cells maintained in human ossicles.

Furthermore, to confirm the presence of functional, self-renewing human HSPCs in human ossicle, secondary transplantation was performed. We isolated hCD34+ cells from mouse bone and human ossicle and transplanted them separately in sublethal irradiated secondary recipients (Figure 2D). After 8 weeks we observed a tendencial higher engraftment of hCD45+ cells in mice transplanted with hCD34+ cells derived from the human ossicle. Interestingly, human ossicle derived CD34+ cells tended to give higher engraftment compared to mouse bone-derived CD34+ cells. At 8 weeks after transplantation, we identified mature B cells, T cells and significant higher levels of Myeloid cells from the human ossicle derived CD34+ cells (Figure 2E). Further, hematopoietic stem and progenitor cells (HSPCs, HSC, MPP and MLP) as well as Myeloid Progenitors (MyelP, CMP/MEP and GMP) derived from human ossicle show a better engraftment than mouse bone derived human HSPCs and MyelP (Figure

2E). This data suggests that the human ossicle provide a better niche to maintain functional and self-renewing human HSPCs as the mouse bone of immune-compromised mice.

It was shown that human MSCs are able to undergo *in vivo* endochondral ossification and are able to build a cavity that supports host derived hematopoietic cells in immunocompromised mouse models. Nevertheless the long standing question is, if these human bone organs can also support human hematopoiesis. In this study we show an ossicle model system that can serve as a niche to maintain human hematopoietic stem cell activity more efficient than existing xenocraft models. We used a standardized and reproducible *in vitro* protocol, that allows an in-process control of the hypertrophic cartiladge before implantation into hTPOhSIRP mice. The human bone organ model was engineered by activation of hMSCs *in vitro* towards an endochondral ossification. At 12 weeks after implantation we could observe a selforganized progression of the process, leading to bone development and efficient vascularization and finally into a functional hematopoietic niche. Nevertheless, further studies are required to validate origin (human/mouse) of the functional niche cells. We showed that human HSPCs injected into the peripheral blood circulation home to human ossicles after whole body subleathal irradiation, suggesting that human HSPCs can migrate in and out of ossicles similar to long bone. However, the impact of irradiation on the newly generated human bone needs to be determined. After irradiation, a stable engraftment of human CD45+ cells was observed in mouse bone and human ossicle. Both lympohiod and myeloid lineage cells were observed by FACS analysis. Interestingly we found that the frequency of myeloid progenitors is less within the human ossicle. In our model, human HSPCs were found to be slightly more quiescent in the human ossicle suggesting that human ossicle are more similar to a human HSC niche than the mouse bone. However no statistical significance was defermined. The most recognized assay to prove functional HSCs activity is the long-term reconstitution assay. In our study we could show that ossicle derived human CD34+ cells show a better reconstitution activity (significant more myeloid cells) compared to mouse bone derived human CD34+ cells. These results were confirmed by a significant better CFU-activtiy of ossicle derived human CD34+ compared to mouse bone derived human CD34+ cells. Some of the previous ossicle studies lack these functional assay. Reinisch et. Al., could show secondary engraftment of ossicle

derived human HSPCs only in one recipient transplanted with ossicle but not in non-ossicle NSG mice.

Most of our understanding of the BM niche and the interaction of HSCs with its environment comes from mouse studies, whereas little is know about human HSCs and their environment, because of the lack of models that mimic the human BM microenvironment. Several studies suggest that human ossicle models can be succefully engrafted with human Leukemia cells and therefore could be used as a modelt o evaluate anti-leukemia drugs[101]. There is increasing evidence that leukemia development is dependent on cellular interactions with the BM niches[137]. As our model mimics a more closly the human niche it could be used to study the effect of the BM microenvironment on leukemia development and progression.

Here we suggest a human bone organ model that more closely resembles a human bone organ and is able to maintain functional human HSCs. Therefore the system can be used to study human healthy as well as malignant hematopoiesis in its environment that can be genetically engineered (e.g. knockin out or overexpression of specific niche factors) and used for therapeutic purposes.

Acknowledgment

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Authorship contribution

K.F., S.P., P.B. and H.T. designed research, performed experiments, analyzed results and wrote the paper. K.F. and S.P. made the figures. T.S., H.T., M.G.M. and I.M. designed research and wrote the paper.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

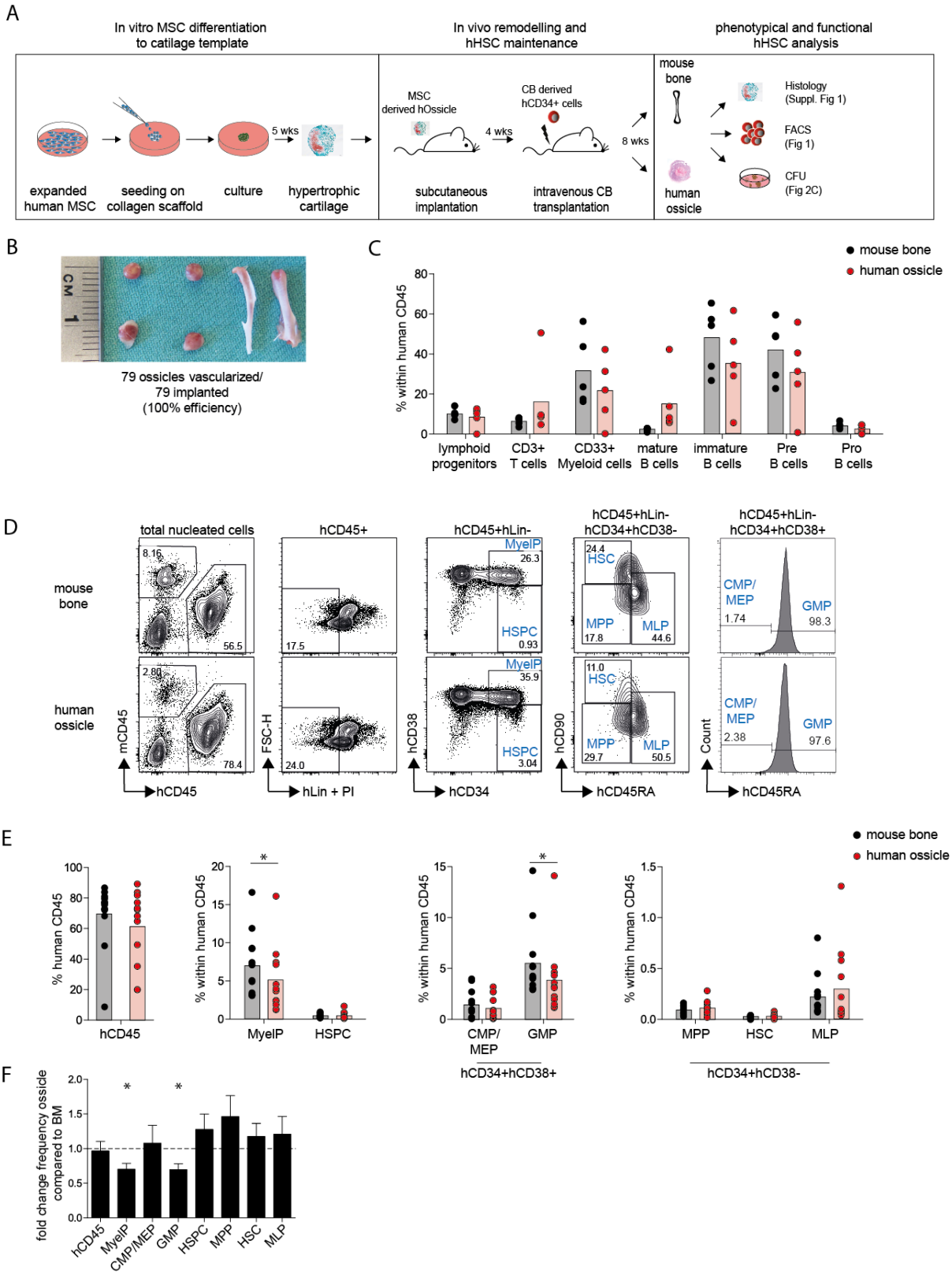


Figure 1 Human bone organ can maintain phenotypic human hematopoietic stem cells. *(A)* Experimental outline: Human Bone marrow-Mesenchymal stem cells (BM-MSCs) where cultured on a collagen scaffold for 5 weeks under chondrogenic and hypertrophic conditions. The hypertrophic cartilage was implanted subcutaneous into Balb/c Rag2 IL2$^{-/-}$ cγ$^{-/-}$ hTPO^{KI} hSIRPα^{Tg} mice (hTPOhSIRP). 4 weeks post

implantation, sublethal irradiated mice carrying ossicles were transplanted with human Cord blood (CB) derived CD34+ cells. After 8 weeks, cells were harvested from human ossicles and mouse bone and analyzed. *(B)* representative photograph of human ossicle and mouse bone (Femur and Tibia) at 12 weeks post implatation (milimeter scale is shown). The human ossicle are filled with blood as indicated by their red color. *(C)* Summary of cytofluorimetric analysis of mature cells. Lympohid progenitors (hCD45+hCD3-hCD33-hCD19-hCD5-), T cells (hCD45+hCD3+), Myeloid cells (hCD45+hCD33+), mature B cells (hCD45+hCD3-hCD33-hCD19+hCD5+), immature B cells (hCD45+hCD3-hCD33-hCD19+hCD5-), Pre B cells (hCD45+hCD3-hCD33-hCD19+hCD5-hCD10+hCD34-) and Pro B cells (hCD45+hCD3-hCD33-hCD19+hCD5-hCD10+hCD34+). Statistical analysis showed no significance. Bar graphs represent means (*n* =5). *(D)* Representative profile of HSCs and progenitors in mouse bone and *in vivo*-remodeled human ossicles 8 weeks after transplantation of human CB-derived CD34+ cells. *(E)* Summary of cytofluorimetric analysis of BM HSCs and progenitors (n =14). Engraftment >10% hCD45+ cells. Statistical significance was determined with students T-Test, *P<0.05. Bar graphs represent means. *(F)* Fold change of HSCs and Progenitors in human ossicle compared to mouse bone. Bar graphs represent means + s.e.m. (*n* =14). Statistical significance was determined with students T-Test, *P<0.05.

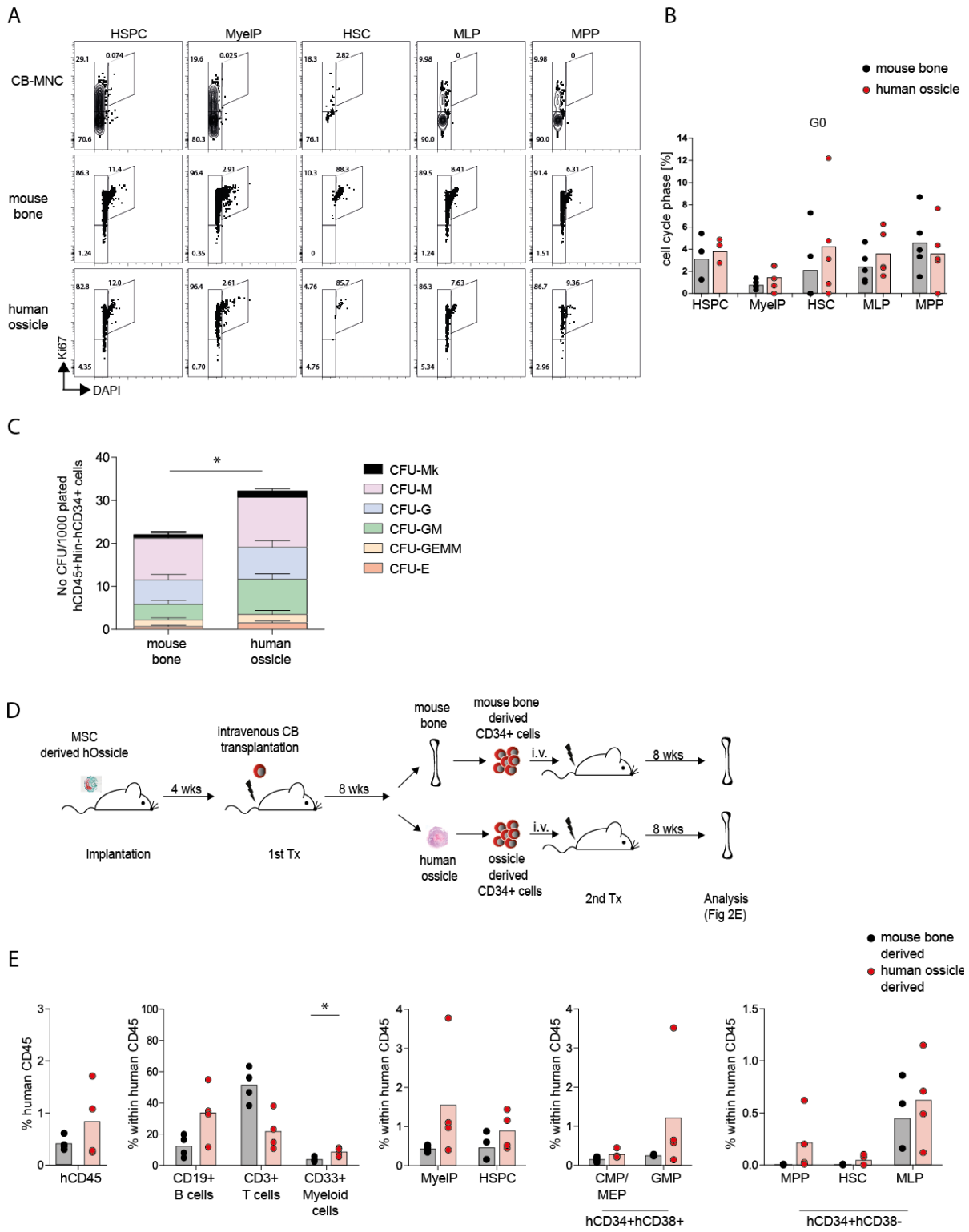
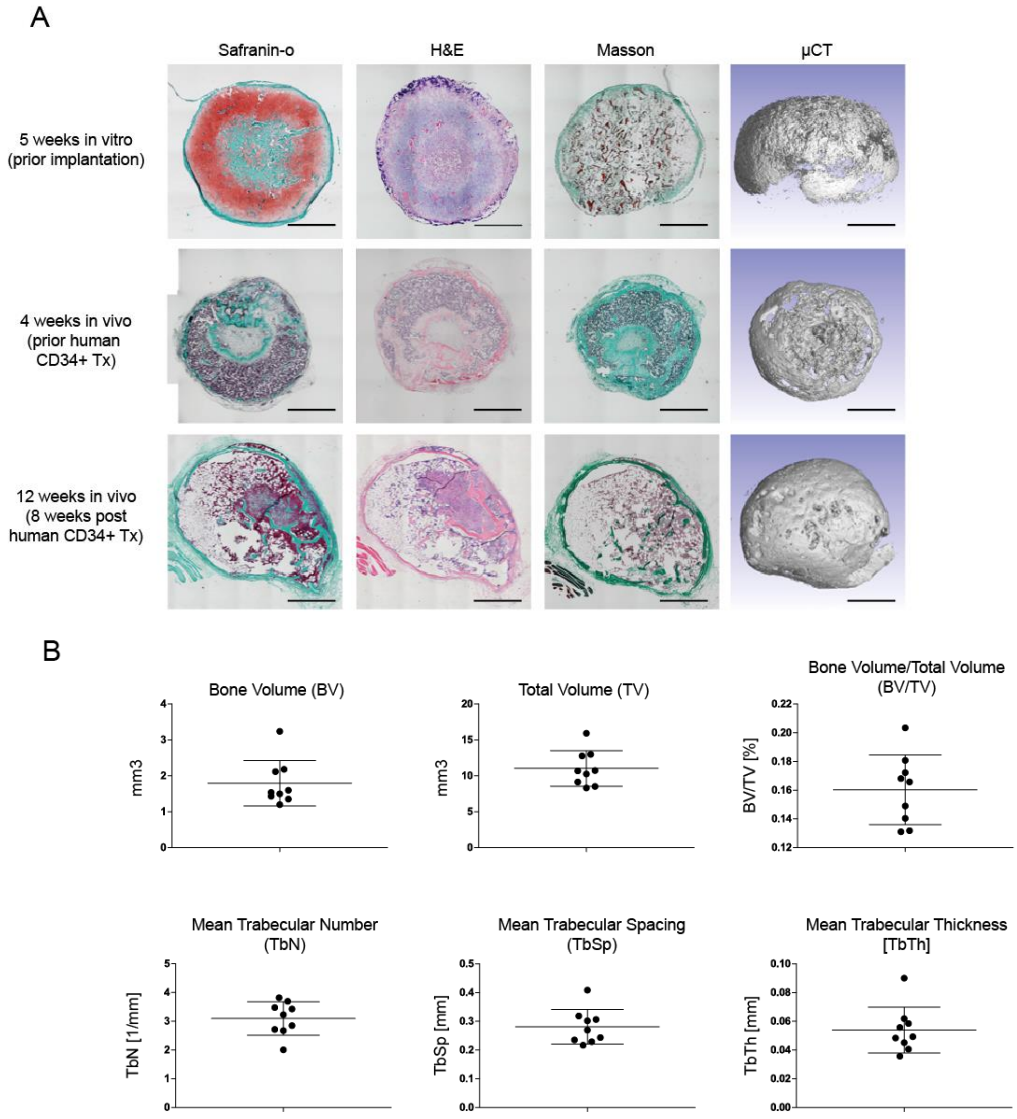


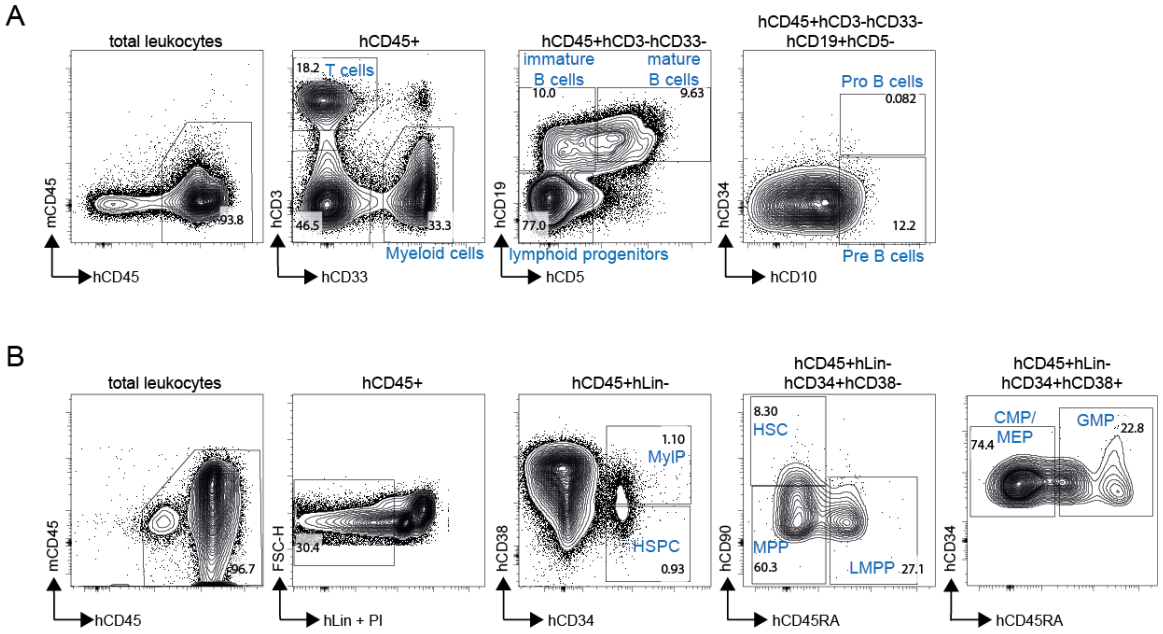
Figure 2 Maintenance of functional human HSCs in the implanted human bone organ. *(A)* Representative profile of cell cycle analysis of human HSCs and progenitors in mouse bone and human ossicle. *(B)* Summary of cytofluorimetric analysis of cell cycle status of HSCs and progenitors in mouse bone and human ossicle 8 weeks after transplantation of human CD34+ cells (n=5). *(C)* Number of CFUs (colony-forming cells) in 1000 plated hCD45+lin-CD34+ cells. Distribution of

myeloid colony subtypes on day 14 . CFU, colony-forming unit; GEMM (granulocyte, erythrocyte, macrophage, megakaryocyte); Mk, megakaryocyte; E, erythrocyte; GM, granulocyte and macrophage; G or M, granulocyte or macrophage. Bar graphs show data from two independent experiments, n=9 ; Statistical significance was determined with two-way ANOVA, *P<0.05. *(D)* Experimental outline: Human bone organs were engineered and implated as decribed before. 8 weeks after transplantation of human CD34+ (1st Tx), cells from mouse bone and human ossicle were harvested, enriched for human CD34+ cells and transplanted into sublethal irradiated hTPOhSIRP mice (2nd Tx). *(E)* Summary of secondary engraftment of human CD34+ HSPC in mouse BM. Purified human CD34+ cells derived from engrafted ossicle and mouse bone were transplanted into sublethal irradiated recipient mice (n=4 from each) and analyzed 8 weeks after transplantation. Statistical significance was determined with students T-test, *P<0.05. Bar graphs represent means.

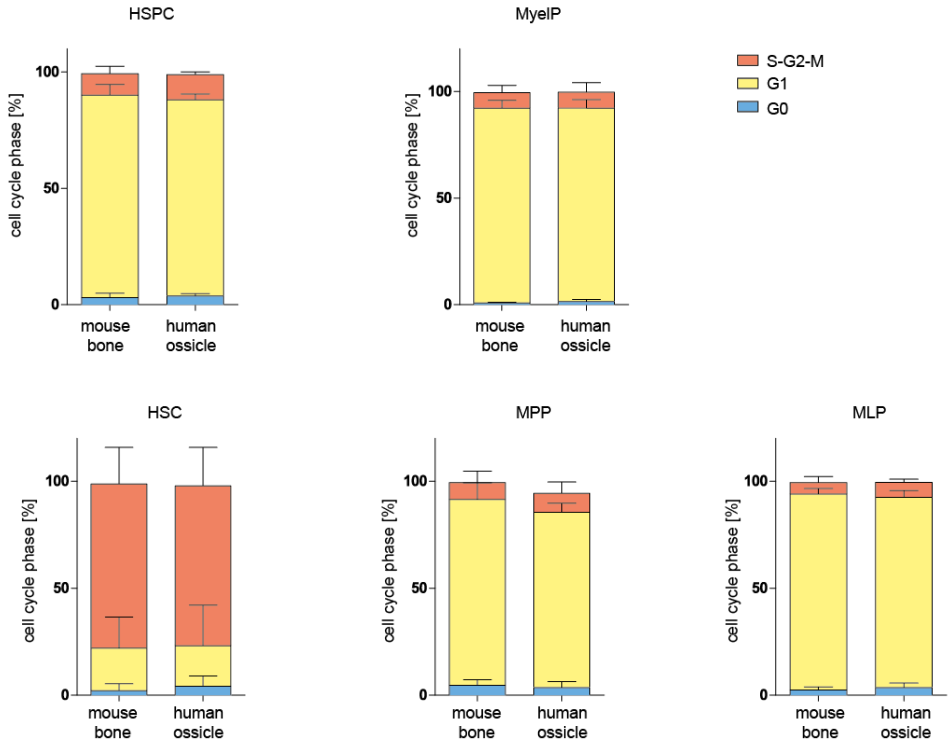
Supplemental Figures



Supplemental Figure 1 Characterization of *in vitro* and *in vivo* bone tissue formation. *(A)* Representative sections (Safranin O, H&E and Masson's Trichrome) and 3D reconstructed microtomographic (µCT) images of samples after 5 weeks *in vitro* culture, 4 weeks after implantation into hTPOhSIRP mice and 12 weeks after implantation (8 weeks after CB CD34+ transplantation). Scale bar 1mm. (B) Quantitative histomorphometric data (n=9) of the total volume of the ossicle (TV) as well as the bone volume within an ossicle (BV) and its ratio (BV/TV). The trabecular bone number (TbN) representing the average number of trabeculae per unit length, their average thickness (TbTh) as well as the mean distance between the trabeculae (TbSp) is represented on the lower graphs.



Supplemental Figure 2 Mature and Human hematopoietic stem and progenitor cells in Cord blood Mononuclear cells. (A) FACS analysis showing the distribution of human mature cells in CB-Mononuclear cells (MNC). Human CD45+ alive cells are gated for mature T cells (hCD45+hCD3+hCD33-) and Myeloid cells (hCD45+hCD3-hCD33+). B cells are divided in mature (hCD45+hCD3-hCD33-hCD19+hCD5+) and immature B cells (hCD45+hCD3-hCD33-hCD19+hCD5-) with Pre (hCD45+hCD3-hCD33-hCD19+hCD5-hCD34-hCD10+) and Pro B cells (hCD45+hCD3-hCD33-hCD19+hCD5-hCD34+hCD10+). (B) FACS analysis showing the distribution of human hematopoietic cells in CB-MNC. Human CD45+, lineage – alive cells are gated for hematopoietic stem and progenitors cells (CD34+CD38-, HSPC) and myeloid Progenitors (CD34+CD38-, MyelP). HSPCs are further subdivided based on CD90 and CD45RA expression into hematopoietic cells (HSC, CD90+CD45RA-), multipotent progenitors (MPP, CD90-CD45RA-) and lymphoid-primed multipotent progenitors (LMPP, CD90+CD45RA+), whereas MyelP are subdivided into common myeloid progenitors and megakaryocyte erythroid progenitors (CMP and MEP, CD34+CD45RA-) and granulocyte macrophage progenitors (GMP, CD34+CD45RA+).



Supplemental Figure 3 Full cell cycle analysis of Hematopoietic stem and progenitor cells from human ossicle and mouse bone. Percentage of each cell cycle phase (G0, G1 and S/G2/M) of HSCs and progenitors from human ossicle and mouse bone 8 weeks after transplantation of human CB-derived CD34+ cells.

CONTRIBUTIONS

In addition to what is going to be published and is planned to be published (listed above in sections research article 1 and 2, I have contributed to various publications and reviews:

a) Research article (in preparation)

Developmental engineering of customized human hematopoietic organs

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This is a project that is based on research article 2 and is an ongoing collaboration with Prof. Timm Schroeder and Prof. Ivan Martin. In this paper I contributed as co-first author.

Abstract

Human Hematopoietic Stem Cells (hHSC) are responsible for the continuous and lifelong replenishment of blood and immune cells. In adult, hHSC reside in the bone marrow (BM) niche, a unique microenvironment tightly orchestrating hHSC functions. However, primary knowledge on the cellular and molecular composition of the human BM niche remains cryptic. Existing insights are predominantly derived from murine studies that do not fully reflect the human system. Here, we report the successful engineering of humanized BM niches as a platform to study human hematopoiesis. Human mesenchymal stromal cells (hMSC) are primed toward developmental program in order to form ectopic bone organs in mice. The resulting human ossicles (hOss) host a human hematopoietic compartment, but also comprised human niche cells derived from the implanted hMSC. In fact, hMSC can be used as cellular vector for the delivery of factors influencing human blood development. Exemplified by the targeted SDF1α delivery, the present study demonstrates the feasibility to design customized humanized hematopoietic organs. The established method is expected to allow gathering human-specific findings of higher translational relevance.

b) Review article (Published: Frontiers in Immunology 2016 Volume 7:502)

Inflamm-Ageing of Hematopoiesis and Hematopoietic Stem Cells

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* These authors contributed equally, co-first authors

This is an on topic review, which I contributed as a co-first author. I was responsible for the inflammation part of this paper.

Abstract

All hematopoietic and immune cells are continuously generated by hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) through highly organized process of stepwise lineage commitment. In the steady state, HSCs are mostly quiescent, while HPCs are actively proliferating and contributing to daily hematopoiesis. In response to hematopoietic challenges, e.g., life-threatening blood loss, infection, and inflammation, HSCs can be activated to proliferate and engage in blood formation. The HSC activation induced by hematopoietic demand is mediated by direct or indirect sensing mechanisms involving pattern recognition receptors or cytokine/chemokine receptors. In contrast to the hematopoietic challenges with obvious clinical symptoms, how the aging process, which involves low-grade chronic inflammation, impacts hematopoiesis remains undefined. Herein, we summarize recent findings pertaining to functional alternations of hematopoiesis, HSCs, and the bone marrow (BM) microenvironment during the processes of aging and inflammation and highlight some common cellular and molecular changes during the processes that influence hematopoiesis and its cells of origin, HSCs and HPCs, as well as the BM microenvironment. We also discuss how age-dependent alterations of the immune system lead to subclinical inflammatory states and how inflammatory signaling might be involved in hematopoietic aging. Our aim is to present evidence supporting the concept of "Inflamm-Aging," or inflammation-associated aging of hematopoiesis.

c) Review article (Published: Haematologica 2016 Volume 101(1):5-19)

Humanized hemato-lymphoid system mice

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This is an on topic review, which I was a contributing author.

Abstract

Over the last decades, incrementally improved xenograft mouse models, supporting the engraftment and development of a human hemato-lymphoid system, have been developed and now represent an important research tool in the field. The most significant contributions made by means of humanized mice are the identification of normal and leukemic hematopoietic stem cells, the characterization of the human hematopoietic hierarchy, and their use as preclinical therapy models for malignant hematopoietic disorders. Successful xenotransplantation depends on three major factors: tolerance by the mouse host, correct spatial location, and appropriately cross-reactive support and interaction factors such as cytokines and major histocompatibility complex molecules. Each of these can be modified. Experimental approaches include the genetic modification of mice to faithfully express human support factors as non-cross-reactive cytokines, to create free niche space, the co-transplantation of human mesenchymal stem cells, the implantation of humanized ossicles or other stroma, and the implantation of human thymic tissue. Besides the source of hematopoietic cells, the conditioning regimen and the route of transplantation also significantly affect human hematopoietic development *in vivo*. We review here the achievements, most recent developments, and the remaining challenges in the generation of pre-clinically-predictive systems for human hematology and immunology, closely resembling the human situation in a xenogeneic mouse environment.

d) Research article (Published: Blood 2016 Volume 128(18):2253-2257)

MPL EXPRESSION ON AML BLASTS PREDICTS PERIPHERAL BLOOD NEUTROPENIA AND THROMBOCYTOPENIA

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I am a contributing author. My contribution was to perform some flowcytometry experiments and real-time PCR.

Abstract

Although the molecular pathways that cause acute myeloid leukemia (AML) are increasingly well understood, the pathogenesis of peripheral blood cytopenia, a major cause of AML mortality, remains obscure. A prevailing assumption states that AML spatially displaces nonleukemic hematopoiesis from the bone marrow. However, examining an initial cohort of 223 AML patients, we found no correlation between bone marrow blast content and cytopenia, questioning the displacement theory. Measuring serum concentration of thrombopoietin (TPO), a key regulator of hematopoietic stem cells and megakaryocytes, revealed loss of physiologic negative correlation with platelet count in AML cases with blasts expressing MPL, the thrombopoietin (scavenging) receptor. Mechanistic studies demonstrated that MPLhi blasts could indeed clear TPO, likely therefore leading to insufficient cytokine levels for nonleukemic hematopoiesis. Microarray analysis in an independent multicenter study cohort of 437 AML cases validated MPL expression as a central predictor of thrombocytopenia and neutropenia in AML. Moreover, t(8;21) AML cases

demonstrated the highest average MPL expression and lowest average platelet and absolute neutrophil counts among subgroups. Our work thus explains the pathophysiology of peripheral blood cytopenia in a relevant number of AML cases.

DISCUSSION AND OUTLOOK

Hematopoietic homeostasis in steady-state and during hematopoietic challenge is maintained by lifelong self-renewing HSCs in the BM. Recent findings have indicated that not only peripheral mature immune cells but also HSCs in BM can respond to infection by sensing pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRR) or to pro-inflammatory cytokines through respective cytokine receptors. However, little is known about its impact on function of lifelong self-renewing HSCs in the BM. In the first part of my thesis I examine how bacterial infection alters HSC functions, and the underlying molecular mechanism, by *in vivo* systematic challenge of LPS, a gram-negative bacterial component, recognized by TLR4. The activation of TLR4 via MyD88 or TRIF pathways during bacterial infection is very well studied in immune cells[138]. However, their role in regulating the BM response during infection or inflammation is poorly understood. Our study shows that in mixed BM chimeric mice a direct activation of TLR4 limits competitive repopulating ability of HSCs via TRIF- but not MYD88-mediated pathways, and increases divisional history and DNA damage and repair responses in HSCs. Pharmacological inhibition of p38 mitogen activated protein kinase (MAPK14) and reactive oxygen species (ROS) rescued HSC dysfunction. This data shows evidence that *in vivo* LPS directly activates BM resident HSC via a TLR4-TRIF-ROS-p38 mediated pathway, and results in limiting their competitive self-renewal possibly through increased divisional history and DNA damage. In line with our study, Zhang et. al.[139] confirmed that HSCs function is altered during sepsis in an TLR4-TRIF dependent manner. However, they also suggest that MYD88 plays a dominant role in myelosuppression during sepsis. This finding points out that MyD88 and TRIF can occur as two independent processes with cell-specific effects. Another study showed that a depletion of p38α in HSPCs leads to defective cell-cycle progression during hematopoietic stress[140]. This might suggests that p38α activation upon LPS stimulation leads to increased cell-cycle activity and finally to proliferation of HSPCs.

Our as well as other studies suggest that systemic infection might cause accumulation of genetic events in HSCs that might lead to HSC deficiency or even to malignant transformation and eventually development of leukemia. In humans, there has been evidences that an increased history of infections or autoimmune diseases lead to a higher risk to develop leukemia later in life[65]. However, most mutations occur with DNA replication during cell division which does not ressecary lead to blood

cancer. One might speculate that in a steady-state environment, these genetically altered HSCs might be removed from the HSC pool or corrected at the DNA level by naturally existing surveillance mechanisms (e.g., apoptosis or DNA repair machinery). A minimum of two to three driver mutations are needed for the development of leukemia. So far it is unknown how mutations that occur during normal hematopoiesis relate to mutations that initiate cancer. We hypothesize that in chronic inflammatory conditions genetically altered HSCs might escape from the normal regulatory mechanisms, i.e. the mutated HSC clones might survive and accumulate further critical genetic events. To prove this hypothesis I have started to establish clonal analysis which allows to determine the number of single nucleotide variations (SNVs) on single cell-derived DNA by whole exome sequencing (WES). We will compare numbers of SNVs in HSCs from animals with the following different HSC challenges: A) HSCs in steady state (6 months repetitive PBS injection), B) HSCs exposed to chronic inflammation (one injection every months for 6 months), C) HSCs that have been maximally expanded by 3 rounds of serial transplantation, D) aged HSCs (2 years old), E) HSCs that are driven into cell cycle through thrombopoietin receptor (c-Mpl) agonist treatment (weekly injection over 2 month and 2 month recovery)[29] and F) HSC that already carry a genetic mutation (JAK2-V617F) which is known to be a driver mutation for myeloproliferative neoplasms [141]. These and other studies will allow to experimentally test the effect of inflammation and inflammation induced proliferation on the occurrence of relevant, possibly HSC to LIC transforming events.

Hematopoietic stem and progenitor cells are maintained in the BM niche, which is very well characterized in mouse. However, little is known about the human BM niche and the interactions of HSCs with its environment. In the recent years, several studies developed different tissue engineering protocols, that are based on the ability of human MSCs to undergo endochondral ossification and develop a bone-like structure *in vivo* (reviewed in [55]). In order to mimic a morphological and functional human BM niche, we took a developmental tissue engineering approach that allows to *ex vivo* generate a human cartilage template with human adult BM-derived MSCs, and to *in vivo* develop human bone organs (thereafter called “ossicles”) through endochondral ossification[99]. After implantation, human MSC-derived ossicles developed a vascular network and a mature trabecular bone-like structure. Flow-

cytometric analysis at 2-month post CB transplantation showed comparable development of human hematopoiesis with phenotypic HSCs in the human ossicles compared to mouse BM. Myeloid-colony forming unit (CFU) assays and serial transplantation showed a significantly higher frequency of hematopoietic stem and progenitor cells maintained in human ossicles than in mouse BM. This suggests that human ossicles can serve as niche to support human hematopoiesis. Our study allows to study human HSCs in an environment that mimics more closely the human BM because of the *in vitro* endochondral differentiation before implantation. In contrast most of the studies directly implant undifferentiated MSC into mice and therefore cannot control their differentiation. The fact that we found immature and mature hematopoietic cells within the ossicle suggests a robust differentiation of human HSPCs within the human environment. The functional assays suggest that the human ossicle might have a local cytokine milieu that is closer to physiological conditions of human bone marrow, as they give rise to more functional HSPCs. These functional assays are not very well studied in the published ossicle models[101] as most of them were designed to increase human engraftment but not model a human BM niche. Several xenotransplantation models have been reported to improve engraftment of human HSCs by modifying human cytokines within immun-compromised mouse models[134,142-144] or co-transplantation of MSCs with HSPCs[145] or transplantation of engineered bioscaffolds[103]. The origin of the different ossicle niche cells still have to be investigated. One can assume that the osteogenic, adipogenic and stroma cells originate from the human donor MSCs while the endothelial cells as well as some hematopoietic cells are recruited from the recipient mouse. The engineering of a heterotrophic human BM niche that is transplantable and genetically re-engineerable will serve as a platform that allows to study physiology and pathophysiology of healthy and diseased human hematopoiesis in their optimized environment *in vivo*. However, the ossicle models in other recent studies do not mimic the morphological and functional features of the human niche[100-102,146]. Therefore, it might be difficult to translate these preclinical findings from bench to bedside. In contrast our studies might suggest a better model to predict results in medicine because of the higher similarity of the human ossicle to a human BM niche.

CONCLUSIONS

1. During Inflammation, direct TLR4 activation induces HSC cycling and impairs their competitive repopulating ability.

2. TLR4-signaling in HSCs is not MyD88 but TRIF-dependent and leads to activation of p38α and generation of ROS.

3. Inhibition of TLR4-TRIF-ROS-p38 signalling prevents LPS-induced HSC dysfunction.

4. Continuous TLR4-stimulation might lead to DNA damage in HSCs.

5. BM-derived human MSCs can form a hematopoietic niche with human hematopoiesis (engineered ossicle).

6. Functional human HSPCs are maintained within the human engineered bone organ in vivo.

7. The human hematopoietic ossicle niche is re-engineerable, serving as a platform to study normal and malignant human hematopoiesis *in vivo*.

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REFERENCE LIST

1 Takizawa, H., Regoes, R. R., Boddupalli, C. S., Bonhoeffer, S. & Manz, M. G. Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. *J Exp Med* **208**, 273-284, doi:10.1084/jem.20101643 (2011).

2 Doulatov, S., Notta, F., Laurenti, E. & Dick, J. E. Hematopoiesis: a human perspective. *Cell Stem Cell* **10**, 120-136, doi:10.1016/j.stem.2012.01.006 (2012).

3 Trumpp, A., Essers, M. & Wilson, A. Awakening dormant haematopoietic stem cells. *Nat Rev Immunol* **10**, 201-209, doi:10.1038/nri2726 (2010).

4 Kondo, M. *et al.* Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* **21**, 759-806, doi:10.1146/annurev.immunol.21.120601.141007 (2003).

5 Gordon, M. Y., Lewis, J. L. & Marley, S. B. Of mice and men...and elephants. *Blood* **100**, 4679-4680, doi:10.1182/blood-2002-08-2517 (2002).

6 Pegg, D. E. A Quantitative Study of Bone Marrow Grafting: Implications for Human Bone Marrow Infusion. *Br J Cancer* **16**, 400-416 (1962).

7 Harrison, W. J. The total cellularity of the bone marrow in man. *J Clin Pathol* **15**, 254-259 (1962).

8 McCulloch, E. A. & Till, J. E. The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. *Radiat Res* **13**, 115-125 (1960).

9 Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. & Dzierzak, E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301 (1994).

10 Medvinsky, A. L., Samoylina, N. L., Muller, A. M. & Dzierzak, E. A. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature* **364**, 64-67, doi:10.1038/364064a0 (1993).

11 Kieusseian, A., Brunet de la Grange, P., Burlen-Defranoux, O., Godin, I. & Cumano, A. Immature hematopoietic stem cells undergo maturation in the fetal liver. *Development* **139**, 3521-3530, doi:10.1242/dev.079210 (2012).

12 Morrison, S. J. & Weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1**, 661-673 (1994).

13 Busch, K. *et al.* Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* **518**, 542-546, doi:10.1038/nature14242 (2015).

14 Passegue, E., Wagers, A. J., Giuriato, S., Anderson, W. C. & Weissman, I. L. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med* **202**, 1599-1611, doi:10.1084/jem.20050967 (2005).

15 Schoedel, K. B. *et al.* The bulk of the hematopoietic stem cell population is dispensable for murine steady-state and stress hematopoiesis. *Blood*, doi:10.1182/blood-2016-03-706010 (2016).

16 Majeti, R., Park, C. Y. & Weissman, I. L. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell* **1**, 635-645, doi:10.1016/j.stem.2007.10.001 (2007).

17 Bender, J. G. *et al.* Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood* **77**, 2591-2596 (1991).

18 Pang, W. W. *et al.* Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc Natl Acad Sci U S A* **108**, 20012-20017, doi:10.1073/pnas.1116110108 (2011).

19 Ema, H. *et al.* Adult mouse hematopoietic stem cells: purification and single-cell assays. *Nat Protoc* **1**, 2979-2987, doi:10.1038/nprot.2006.447 (2006).

20 Abkowitz, J. L., Catlin, S. N., McCallie, M. T. & Guttorp, P. Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. *Blood* **100**, 2665-2667, doi:10.1182/blood-2002-03-0822 (2002).

21 Pietras, E. M., Warr, M. R. & Passegue, E. Cell cycle regulation in hematopoietic stem cells. *J Cell Biol* **195**, 709-720, doi:10.1083/jcb.201102131 (2011).

22 Capel, B., Hawley, R., Covarrubias, L., Hawley, T. & Mintz, B. Clonal contributions of small numbers of retrovirally marked hematopoietic stem cells engrafted in unirradiated neonatal W/Wv mice. *Proc Natl Acad Sci U S A* **86**, 4564-4568 (1989).

23 Lemischka, I. R., Raulet, D. H. & Mulligan, R. C. Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* **45**, 917-927 (1986).

24 Sun, J. *et al.* Clonal dynamics of native haematopoiesis. *Nature* **514**, 322-327, doi:10.1038/nature13824 (2014).
25 Wilson, A. *et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* **135**, 1118-1129, doi:10.1016/j.cell.2008.10.048 (2008).
26 Essers, M. A. *et al.* IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908, doi:10.1038/nature07815 (2009).
27 Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. & Goodell, M. A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* **465**, 793-797, doi:10.1038/nature09135 (2010).
28 Morrison, S. J., Wright, D. E. & Weissman, I. L. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci U S A* **94**, 1908-1913 (1997).
29 Kovtonyuk, L. V., Manz, M. G. & Takizawa, H. Enhanced thrombopoietin but not G-CSF receptor stimulation induces self-renewing hematopoietic stem cell divisions in vivo. *Blood* **127**, 3175-3179, doi:10.1182/blood-2015-09-669929 (2016).
30 Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* **4**, 7-25 (1978).
31 Chow, A. *et al.* Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med* **208**, 261-271, doi:10.1084/jem.20101688 (2011).
32 Winkler, I. G. *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116**, 4815-4828, doi:10.1182/blood-2009-11-253534 (2010).
33 Bruns, I. *et al.* Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat Med* **20**, 1315-1320, doi:10.1038/nm.3707 (2014).
34 Zhao, M. *et al.* Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. *Nat Med* **20**, 1321-1326, doi:10.1038/nm.3706 (2014).
35 Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* **460**, 259-263, doi:10.1038/nature08099 (2009).
36 Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841, doi:10.1038/nature02041 (2003).
37 Calvi, L. M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-846, doi:10.1038/nature02040 (2003).
38 Mansour, A. *et al.* Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med* **209**, 537-549, doi:10.1084/jem.20110994 (2012).
39 Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149-161, doi:10.1016/j.cell.2004.07.004 (2004).
40 Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231-235, doi:10.1038/nature11885 (2013).
41 Ono, N. *et al.* Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. *Dev Cell* **29**, 330-339, doi:10.1016/j.devcel.2014.03.014 (2014).
42 Yamazaki, S. *et al.* Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* **147**, 1146-1158, doi:10.1016/j.cell.2011.09.053 (2011).
43 Mendez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829-834, doi:10.1038/nature09262 (2010).
44 Omatsu, Y. *et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* **33**, 387-399, doi:10.1016/j.immuni.2010.08.017 (2010).
45 Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457-462, doi:10.1038/nature10783 (2012).
46 Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121, doi:10.1016/j.cell.2005.05.026 (2005).
47 Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-988, doi:10.1016/j.immuni.2006.10.016 (2006).
48 Nombela-Arrieta, C. *et al.* Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol* **15**, 533-543, doi:10.1038/ncb2730 (2013).

49 Arai, F. & Suda, T. Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann N Y Acad Sci* **1106**, 41-53, doi:10.1196/annals.1392.005 (2007).
50 Hooper, A. T. *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* **4**, 263-274, doi:10.1016/j.stem.2009.01.006 (2009).
51 Gori, J. L. *et al.* Endothelial Cells Promote Expansion of Long-Term Engrafting Marrow Hematopoietic Stem and Progenitor Cells in Primates. *Stem Cells Transl Med*, doi:10.5966/sctm.2016-0240 (2016).
52 Guezguez, B. *et al.* Regional localization within the bone marrow influences the functional capacity of human HSCs. *Cell Stem Cell* **13**, 175-189, doi:10.1016/j.stem.2013.06.015 (2013).
53 Pinho, S. *et al.* PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med* **210**, 1351-1367, doi:10.1084/jem.20122252 (2013).
54 Chang, M. K. *et al.* Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *J Immunol* **181**, 1232-1244 (2008).
55 Theocharides, A. P., Rongvaux, A., Fritsch, K., Flavell, R. A. & Manz, M. G. Humanized hemato-lymphoid system mice. *Haematologica* **101**, 5-19, doi:10.3324/haematol.2014.115212 (2016).
56 Sadik, C. D., Kim, N. D. & Luster, A. D. Neutrophils cascading their way to inflammation. *Trends in immunology* **32**, 452-460, doi:10.1016/j.it.2011.06.008 (2011).
57 Shi, C. & Pamer, E. G. Monocyte recruitment during infection and inflammation. *Nature reviews. Immunology* **11**, 762-774, doi:10.1038/nri3070 (2011).
58 Takizawa, H., Boettcher, S. & Manz, M. G. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* **119**, 2991-3002, doi:10.1182/blood-2011-12-380113 (2012).
59 Nagai, Y. *et al.* Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity* **24**, 801-812, doi:10.1016/j.immuni.2006.04.008 (2006).
60 Massberg, S. *et al.* Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell* **131**, 994-1008, doi:10.1016/j.cell.2007.09.047 (2007).
61 Schmid, M. A., Takizawa, H., Baumjohann, D. R., Saito, Y. & Manz, M. G. Bone marrow dendritic cell progenitors sense pathogens via Toll-like receptors and subsequently migrate to inflamed lymph nodes. *Blood* **118**, 4829-4840, doi:10.1182/blood-2011-03-344960 (2011).
62 Sato, T. *et al.* Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. *Nat Med* **15**, 696-700, doi:10.1038/nm.1973 (2009).
63 Zhao, J. L. *et al.* Conversion of danger signals into cytokine signals by hematopoietic stem and progenitor cells for regulation of stress-induced hematopoiesis. *Cell Stem Cell* **14**, 445-459, doi:10.1016/j.stem.2014.01.007 (2014).
64 Walter, D. *et al.* Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature* **520**, 549-552, doi:10.1038/nature14131 (2015).
65 Kristinsson, S. Y. *et al.* Chronic immune stimulation might act as a trigger for the development of acute myeloid leukemia or myelodysplastic syndromes. *J Clin Oncol* **29**, 2897-2903, doi:10.1200/JCO.2011.34.8540 (2011).
66 Kristinsson, S. Y., Landgren, O., Samuelsson, J., Bjorkholm, M. & Goldin, L. R. Autoimmunity and the risk of myeloproliferative neoplasms. *Haematologica* **95**, 1216-1220, doi:10.3324/haematol.2009.020412 (2010).
67 Welch, J. S. *et al.* The origin and evolution of mutations in acute myeloid leukemia. *Cell* **150**, 264-278, doi:10.1016/j.cell.2012.06.023
S0092-8674(12)00777-5 [pii] (2012).
68 Xie, M. *et al.* Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* **20**, 1472-1478, doi:10.1038/nm.3733 (2014).
69 Opitz, C. A. *et al.* Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-beta and protein kinase R. *Stem Cells* **27**, 909-919, doi:10.1002/stem.7 (2009).
70 Andonegui, G. *et al.* Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. *J Clin Invest* **119**, 1921-1930 (2009).
71 Day, R. B., Bhattacharya, D., Nagasawa, T. & Link, D. C. Granulocyte colony-stimulating factor reprograms bone marrow stromal cells to actively suppress B lymphopoiesis in mice. *Blood* **125**, 3114-3117, doi:10.1182/blood-2015-02-629444 (2015).

72 Bunnell, B. A., Betancourt, A. M. & Sullivan, D. E. New concepts on the immune modulation mediated by mesenchymal stem cells. *Stem Cell Res Ther* **1**, 34, doi:10.1186/scrt34 (2010).
73 Martino, M. M. *et al.* Inhibition of IL-1R1/MyD88 signalling promotes mesenchymal stem cell-driven tissue regeneration. *Nat Commun* **7**, 11051, doi:10.1038/ncomms11051 (2016).
74 Zhang, W. Q. *et al.* Regulation of human mesenchymal stem cell differentiation by TREM-2. *Hum Immunol* **77**, 476-482, doi:10.1016/j.humimm.2015.06.005 (2016).
75 Shi, C. *et al.* Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity* **34**, 590-601, doi:10.1016/j.immuni.2011.02.016 (2011).
76 Ziegler, P., Boettcher, S., Takizawa, H., Manz, M. G. & Brummendorf, T. H. LPS-stimulated human bone marrow stroma cells support myeloid cell development and progenitor cell maintenance. *Ann Hematol* **95**, 173-178, doi:10.1007/s00277-015-2550-5 (2016).
77 Boettcher, S. *et al.* Cutting edge: LPS-induced emergency myelopoiesis depends on TLR4-expressing nonhematopoietic cells. *J Immunol* **188**, 5824-5828, doi:10.4049/jimmunol.1103253 (2012).
78 Boettcher, S. *et al.* Endothelial cells translate pathogen signals into G-CSF-driven emergency granulopoiesis. *Blood* **124**, 1393-1403, doi:10.1182/blood-2014-04-570762 (2014).
79 Christopher, M. J., Liu, F., Hilton, M. J., Long, F. & Link, D. C. Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. *Blood* **114**, 1331-1339, doi:10.1182/blood-2008-10-184754 (2009).
80 Eash, K. J., Greenbaum, A. M., Gopalan, P. K. & Link, D. C. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest* **120**, 2423-2431, doi:10.1172/JCI41649 (2010).
81 Sieff, C. A., Tsai, S. & Faller, D. V. Interleukin 1 induces cultured human endothelial cell production of granulocyte-macrophage colony-stimulating factor. *J Clin Invest* **79**, 48-51, doi:10.1172/JCI112806 (1987).
82 Broudy, V. C., Kaushansky, K., Segal, G. M., Harlan, J. M. & Adamson, J. W. Tumor necrosis factor type alpha stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A* **83**, 7467-7471 (1986).
83 Schurch, C. M., Riether, C. & Ochsenbein, A. F. Cytotoxic CD8+ T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. *Cell Stem Cell* **14**, 460-472, doi:10.1016/j.stem.2014.01.002 (2014).
84 Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**, 730-737 (1997).
85 Friedenstein, A. J., Gorskaja, J. F. & Kulagina, N. N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* **4**, 267-274 (1976).
86 Beresford, J. N., Bennett, J. H., Devlin, C., Leboy, P. S. & Owen, M. E. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J Cell Sci* **102 (Pt 2)**, 341-351 (1992).
87 Friedenstein, A. J., Chailakhyan, R. K. & Gerasimov, U. V. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* **20**, 263-272 (1987).
88 Howlett, C. R. *et al.* Mineralization in in vitro cultures of rabbit marrow stromal cells. *Clin Orthop Relat Res*, 251-263 (1986).
89 Mardon, H. J., Bee, J., von der Mark, K. & Owen, M. E. Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. *Cell Tissue Res* **250**, 157-165 (1987).
90 Owen, M. & Friedenstein, A. J. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* **136**, 42-60 (1988).
91 Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147 (1999).
92 Wakitani, S., Saito, T. & Caplan, A. I. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* **18**, 1417-1426, doi:10.1002/mus.880181212 (1995).
93 Kuznetsov, S. A. *et al.* Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J Bone Miner Res* **12**, 1335-1347, doi:10.1359/jbmr.1997.12.9.1335 (1997).
94 Horwitz, E. M. & Keating, A. Nonhematopoietic mesenchymal stem cells: what are they? *Cytotherapy* **2**, 387-388 (2000).
95 Park, D. *et al.* Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* **10**, 259-272, doi:10.1016/j.stem.2012.02.003

S1934-5909(12)00061-6 [pii] (2012).
96 Scotti, C. *et al.* Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 7251-7256, doi:10.1073/pnas.1000302107 (2010).
97 Chan, C. K. *et al.* Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* **457**, 490-494, doi:10.1038/nature07547 (2009).
98 Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324-336, doi:10.1016/j.cell.2007.08.025 (2007).
99 Scotti, C. *et al.* Engineering of a functional bone organ through endochondral ossification. *Proc Natl Acad Sci U S A* **110**, 3997-4002, doi:10.1073/pnas.1220108110 (2013).
100 Reinisch, A. *et al.* Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. *Blood* **125**, 249-260, doi:10.1182/blood-2014-04-572255 (2015).
101 Reinisch, A. *et al.* A humanized bone marrow ossicle xenotransplantation model enables improved engraftment of healthy and leukemic human hematopoietic cells. *Nat Med* **22**, 812-821, doi:10.1038/nm.4103 (2016).
102 Chen, Y. *et al.* Human extramedullary bone marrow in mice: a novel in vivo model of genetically controlled hematopoietic microenvironment. *Blood* **119**, 4971-4980, doi:10.1182/blood-2011-11-389957 (2012).
103 Holzapfel, B. M. *et al.* Tissue engineered humanized bone supports human hematopoiesis in vivo. *Biomaterials* **61**, 103-114, doi:10.1016/j.biomaterials.2015.04.057 (2015).
104 Via, A. G., Frizziero, A. & Oliva, F. Biological properties of mesenchymal Stem Cells from different sources. *Muscles Ligaments Tendons J* **2**, 154-162 (2012).
105 Orkin, S. H. & Zon, L. I. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132**, 631-644, doi:10.1016/j.cell.2008.01.025 (2008).
106 Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* **505**, 327-334, doi:10.1038/nature12984 (2014).
107 Burberry, A. *et al.* Infection mobilizes hematopoietic stem cells through cooperative NOD-like receptor and Toll-like receptor signaling. *Cell Host Microbe* **15**, 779-791, doi:10.1016/j.chom.2014.05.004 (2014).
108 Manz, M. G. & Boettcher, S. Emergency granulopoiesis. *Nat Rev Immunol* **14**, 302-314, doi:10.1038/nri3660 (2014).
109 Liu, A. *et al.* Cutting Edge: Hematopoietic Stem Cell Expansion and Common Lymphoid Progenitor Depletion Require Hematopoietic-Derived, Cell-Autonomous TLR4 in a Model of Chronic Endotoxin. *J Immunol* **195**, 2524-2528, doi:10.4049/jimmunol.1501231 (2015).
110 Morita, Y., Ema, H. & Nakauchi, H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med* **207**, 1173-1182, doi:10.1084/jem.20091318 (2010).
111 Cheshier, S. H., Morrison, S. J., Liao, X. & Weissman, I. L. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A* **96**, 3120-3125 (1999).
112 Chen, C., Liu, Y., Liu, Y. & Zheng, P. Mammalian target of rapamycin activation underlies HSC defects in autoimmune disease and inflammation in mice. *J Clin Invest* **120**, 4091-4101, doi:10.1172/JCI43873 (2010).
113 Esplin, B. L. *et al.* Chronic exposure to a TLR ligand injures hematopoietic stem cells. *J Immunol* **186**, 5367-5375, doi:10.4049/jimmunol.1003438 (2011).
114 O'Neill, L. A. & Bowie, A. G. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* **7**, 353-364, doi:10.1038/nri2079 (2007).
115 Ito, K. *et al.* Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* **12**, 446-451, doi:10.1038/nm1388 (2006).
116 Kopp, E. & Ghosh, S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**, 956-959 (1994).
117 Jimi, E. *et al.* Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. *Nat Med* **10**, 617-624, doi:10.1038/nm1054 (2004).
118 Matsuzawa, A. *et al.* ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. *Nat Immunol* **6**, 587-592, doi:10.1038/ni1200 (2005).
119 Zhang, X., Sejas, D. P., Qiu, Y., Williams, D. A. & Pang, Q. Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence. *J Cell Sci* **120**, 1572-1583, doi:10.1242/jcs.003152 (2007).

120 Blanpain, C., Mohrin, M., Sotiropoulou, P. A. & Passegue, E. DNA-damage response in tissue-specific and cancer stem cells. *Cell Stem Cell* **8**, 16-29, doi:10.1016/j.stem.2010.12.012 (2011).
121 Ciccia, A. & Elledge, S. J. The DNA damage response: making it safe to play with knives. *Mol Cell* **40**, 179-204, doi:10.1016/j.molcel.2010.09.019 (2010).
122 Flach, J. *et al.* Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature* **512**, 198-202, doi:10.1038/nature13619 (2014).
123 Dong, Y. *et al.* H2AX phosphorylation regulated by p38 is involved in Bim expression and apoptosis in chronic myelogenous leukemia cells induced by imatinib. *Apoptosis* **19**, 1281-1292, doi:10.1007/s10495-014-0997-9 (2014).
124 Tothova, Z. *et al.* FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* **128**, 325-339, doi:10.1016/j.cell.2007.01.003 (2007).
125 Yahata, T. *et al.* Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood* **118**, 2941-2950, doi:10.1182/blood-2011-01-330050 (2011).
126 Baudet, A. *et al.* RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion. *Blood* **119**, 6255-6258, doi:10.1182/blood-2012-01-403949 (2012).
127 Wang, Y., Liu, L. & Zhou, D. Inhibition of p38 MAPK attenuates ionizing radiation-induced hematopoietic cell senescence and residual bone marrow injury. *Radiat Res* **176**, 743-752 (2011).
128 Oh, J., Lee, Y. D. & Wagers, A. J. Stem cell aging: mechanisms, regulators and therapeutic opportunities. *Nat Med* **20**, 870-880, doi:10.1038/nm.3651 (2014).
129 Drowley, L. *et al.* Cellular antioxidant levels influence muscle stem cell therapy. *Mol Ther* **18**, 1865-1873, doi:10.1038/mt.2010.160 (2010).
130 Bernet, J. D. *et al.* p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice. *Nat Med* **20**, 265-271, doi:10.1038/nm.3465 (2014).
131 Beerman, I. *et al.* Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. *Cell Stem Cell* **12**, 413-425, doi:10.1016/j.stem.2013.01.017 (2013).
132 Sinha, M. *et al.* Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. *Science* **344**, 649-652, doi:10.1126/science.1251152 (2014).
133 Nakamura-Ishizu, A., Takubo, K., Fujioka, M. & Suda, T. Megakaryocytes are essential for HSC quiescence through the production of thrombopoietin. *Biochem Biophys Res Commun* **454**, 353-357, doi:10.1016/j.bbrc.2014.10.095 (2014).
134 Rongvaux, A. *et al.* Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. *Proc Natl Acad Sci U S A* **108**, 2378-2383, doi:10.1073/pnas.1019524108 (2011).
135 Strowig, T. *et al.* Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice. *Proc Natl Acad Sci U S A* **108**, 13218-13223, doi:10.1073/pnas.1109769108 (2011).
136 Shima, H. *et al.* Acquisition of G(0) state by CD34-positive cord blood cells after bone marrow transplantation. *Exp Hematol* **38**, 1231-1240, doi:10.1016/j.exphem.2010.08.004 (2010).
137 Sanchez-Aguilera, A. & Mendez-Ferrer, S. The hematopoietic stem-cell niche in health and leukemia. *Cell Mol Life Sci*, doi:10.1007/s00018-016-2306-y (2016).
138 Weighardt, H. & Holzmann, B. Role of Toll-like receptor responses for sepsis pathogenesis. *Immunobiology* **212**, 715-722, doi:10.1016/j.imbio.2007.09.010 (2007).
139 Zhang, H. *et al.* Sepsis Induces Hematopoietic Stem Cell Exhaustion and Myelosuppression through Distinct Contributions of TRIF and MYD88. *Stem Cell Reports* **6**, 940-956, doi:10.1016/j.stemcr.2016.05.002 (2016).
140 Karigane, D. *et al.* p38alpha Activates Purine Metabolism to Initiate Hematopoietic Stem/Progenitor Cell Cycling in Response to Stress. *Cell Stem Cell* **19**, 192-204, doi:10.1016/j.stem.2016.05.013 (2016).
141 Lundberg, P. *et al.* Myeloproliferative neoplasms can be initiated from a single hematopoietic stem cell expressing JAK2-V617F. *J Exp Med* **211**, 2213-2230, doi:10.1084/jem.20131371 (2014).
142 Rongvaux, A. *et al.* Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol* **32**, 364-372, doi:10.1038/nbt.2858 (2014).
143 Willinger, T. *et al.* Human IL-3/GM-CSF knock-in mice support human alveolar macrophage development and human immune responses in the lung. *Proc Natl Acad Sci U S A* **108**, 2390-2395, doi:10.1073/pnas.1019682108 (2011).

144 Willinger, T., Rongvaux, A., Strowig, T., Manz, M. G. & Flavell, R. A. Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement. *Trends Immunol* **32**, 321-327, doi:10.1016/j.it.2011.04.005 (2011).
145 Medyouf, H. *et al.* Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell Stem Cell* **14**, 824-837, doi:10.1016/j.stem.2014.02.014 (2014).
146 Groen, R. W. *et al.* Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16, doi:10.1182/blood-2012-03-414920 (2012).

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11.2012 – present	**Ph.D.** in Cancer Biology, University of Zurich, Life Science Graduate School, Switzerland
09.2010 – 09.2012	**M.Sc.** Molecular and Cell Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany
09.2007 – 09.2012	**B.Sc.** Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany
08.1999 – 07.2007	Samuel-von-Pufendorf Gymnasium, Flöha, Germany

Research

11.2012 – present Laboratory of Experimental Hematology, Division of Hematology, University Hospital Zurich and University Zurich, Switzerland.
Dissertation:
Supervision: Prof. Dr. med. Markus G. Manz

04.2015 – 05.2015 International Research Center for Medical Sciences, Kumamoto University, Japan.
Project: Analysis of human bone marrow niche using novel humanized mice
Supervision: Dr. Hitoshi Takizawa

09.2010 – 09.2012 Division of Molecular Immunology, Nikolaus Fiebiger Zentrum, Department of Internal Medicine III, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany.
M.Sc. thesis project: Determination of the integration site of Swiprosin-2/EFhd1 in transgenic mice and characterization of Swiprosin-2/EFhd1 transgenic mice.
Supervision: Prof. Dr. Hans-Martin Jäck

09.2007 – 09.2012 Division of Molecular Immunology, Nikolaus Fiebiger Zentrum, Department of Internal Medicine III, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany.
B.Sc. thesis project: Expression analysis of Swiprosin-1/EFhd2
Supervision: Prof. Dr. Hans-Martin Jäck

07.2009 – 08.2009 Department of Immunology, School of Medicine, Tongji University, Shanghai, China.
project: Cloning, expression and functional studies on SR-A and Lox-1 receptor.
Supervision:

07.2008 – 09.2008 Department of Human Genetics, Helmholtz Centre Munich, Germany.
project: Cloning, Yeast-Two-Hybrid System
Supervision:

Publications

1. Larisa V. Kovtonyuk, **Kristin Fritsch**, Markus G. Manz, Hitoshi Takizawa., *Inflamm-Ageing of Hematopoiesis and Hematopoietic Stem Cells*, Frontiers in Immunology 2016
2. Alexandre P.A. Theocharides, Anthony Rongvaux, **Kristin Fritsch**, Richard A. Flavell, and Markus G. Manz., *Humanized hemato-lymphoid system mice*, Haematologica 2016.
3. Philipp J. Rauch, Jana M. Ellegast, Corinne C. Widmer, **Kristin Fritsch**, Jeroen S. Goede, Peter J.M. Valk, Bob Löwenberg, Hitoshi Takizawa, & Markus G. Manz., *MPL EXPRESSION ON AML BLASTS PREDICTS PERIPHERAL BLOOD NEUTROPENIA AND THROMBOCYTOPENIA*, Blood 2016.

Invited speaker

1. 13th Day of Clinical Research, “Maintenance of human hematopoiesis and hematopoietic stem cells in *in vivo* engineered bone organs”, 12.06.2014, Zurich, Switzerland
2. 14th Day of Clinical Research, “*In vivo* maintenance of human hematopoietic stem cells and hematopoiesis by engineered bone organs”, 09.04.2015, Zurich, Switzerland.
3. 15th Day of Clinical Research, “Maintenance of human hematopoiesis in *in vivo* engineered bone organs”, 12.04.2016, Zurich, Switzerland.
4. 1. Frühjahrsversammlung der Schweizerischen Gesellschaft für Allgemeine Innere Medizin (SGAIM) und der Schweizerischen Gesellschaft für Hämatologie (SGH), „*In vivo* maintenance of human hematopoietic stem cells and hematopoiesis by engineered bone organs“, 25. – 27.05.2016, Basel, Switzerland.

Congress poster presentations

1. 5th Cancer Biology PhD student retreat, “Molecular mechanism of hematopoietic stem cell dysfunction induced by chronic lipopolysaccharide challenge”, 12. - 14.02.2014, Filzbach, Switzerland.
2. 10th SSCN Annual Meeting, “*In vivo* maintenance of human hematopoietic stem cells and hematopoiesis by engineered bone organs”, 23.04.2014, Geneva, Switzerland.
3. EMBO Conference Stem cells in cancer and regenerative medicine, “Impact of inflammation on hematopoietic stem cells”, 09. – 12.10.2014, Heidelberg, Germany.
4. 12th Charles Rodolphe Brupbacher Symposium Breakthroughs in Cancer Research and Therapy, “Maintenance of human hematopoietic stem cells and hematopoiesis in *in vivo* engineered bone organs”, 28. – 30.01.2015, Zurich, Switzerland.

5. 6th Cancer Network Zurich retreat, “Maintenance of human hematopoietic stem cells and hematopoiesis in *in vivo* engineered bone organs”, 12. - 14.04.2015, Emetten, Switzerland
6. 14th Day of Clinical Research, “*In vivo* maintenance of human hematopoietic stem cells and hematopoiesis by engineered bone organs”, 09.04.2015, Zurich, Switzerland.
7. VII. Else Kröner-Fresenius Symposium on Adult Stem Cells in Aging, Diseases, and Cancer , “Direct Sensing of Lipopolysaccharide via TLR4-TRIF-ROS-p38 Pathways Impairs Hematopoietic Stem Cell Selfrenewal”, May 31.05. – 03.06.2015, Erice, Sicily, Italy.
8. 5th International Workshop on Humanized Mice, “Maintenance of human hematopoiesis in *in vivo* engineered bone organs”, 28. – 30.01.2016, Zurich, Switzerland.
9. 15th Day of Clinical Research, “Maintenance of human hematopoiesis in *in vivo* engineered bone organs”, 12.04.2016, Zurich, Switzerland.
10. 45th Annual Scientific Meeting ISEH, “Engineered human bone organs maintain human hematopoiesis *in vivo*”, 25. – 28.08.2016, San Diego, USA.

Merits

Poster prize, EMBL Conference 2014, Heidelberg, Germany.
Poster prize, SSCN 2013, Geneva, Switzerland.

Teaching activities

2012 – 2015 Teaching and organizing courses on Flow Cytometry for beginners (MD-PhD students).

Technical expertise

Flow Cytometry analysis and cell sorting, confocal microscopy, primary cell culture and colony forming assay, mouse handling, Hematopoietic cell transplantation, bone engineering.

Software skills

MS Office, Adobe Photoshop, Adobe Illustrator, FlowJo, GraphPad Prism, Imaris.

Language skills

English (fluent), German (native).

Administrative responsibilities

Organisation and writing of animal experimental licenses in order to obtain permission from Veterinary Office to perform experiments.